Mechanisms and drivers of epithelial to mesenchymal transition in COPD

by

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Declaration of originality

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Statement of ethical conduct

Recruitment and collection of samples was approved by the Tasmanian Health and Medical Human Research Ethics Committee (H0013051).

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Abstract

Chronic obstructive pulmonary disease (COPD) is a progressive and irreversible limitation of airflow which predominantly affectsa smokers. One of the mechanisms behind the development and progression of COPD is thought to be epithelial-mesenchymal transition (EMT). EMT is a process whereby epithelial cells, which normally act as the body's primary means of defence against injury, external pathogens and other damaging factors, lose their ability to form a cohesive barrier and instead take on characteristics of a motile, fibrotic and mesenchymal phenotype.

There is *in vitro* evidence that cigarette smoke extract (CSE) can directly induce EMT through production of transforming growth factor-β1 (TGF-β). However, isolated cells in these studies are typically sourced from people with established COPD or with lung cancer. It is not known whether this CSE-TGF-β-EMT pathway occurs at earlier stages of the disease, for example in smokers with normal lung function or in pre-COPD airflow obstruction. Also unclear is whether current therapies for COPD affect the CSE-TGF-β-EMT pathway and if they have beneficial effects early in the development of the disease.

The aims of this study were to investigate the CSE-TGF-β-EMT pathway in isolated epithelial cells from healthy non-smokers, smokers with normal lung function and people with airflow limitation which does not reach the threshold for clinically diagnosed COPD. In doing so, this study attempted to determine whether EMT occurs at very early disease stages, perhaps even before manifestation of clinically significant airflow limitation, and to investigate the effects of CSE on healthy cells from non-smokers. At the same time, the effects of three current COPD therapies - a long acting β-agonist (salmeterol xinafoate), a long acting muscarinic agonist (tiotropium bromide) and a corticosteroid (fluticasone

proprionate) – on both early disease stages and the CSE-TGF-β-EMT pathway were investigated.

Two cell culture models were examined: primary bronchial epithelial cells (pHBECs) and immortalised BEAS-2B airway epithelial cells. The models were tested for expression of EMT markers, as well as markers for the EMT-associated TGF-β and TWIST signalling pathways, using real-time qPCR, immunocytochemistry/histochemistry and ELISA.

Cells from non-smokers, current smokers with normal lung function and both smokers and ex-smokers with airflow limitation were shown to survive both isolation and cryopreservation. The protein expression of EMT markers in the cultured cells agreed with the signals seen in biopsies from the same groups, indicating that phenotype was maintained in culture. However, cells from people with chronic airflow limitation did not show evidence of active EMT, either in culture or in the biopsies, when compared to cells from non-smokers or smokers with normal lung function.

Despite BEAS-2B cells being widely used to study healthy bronchial epithelial cells when primary cells are unavailable, this study demonstrated that the immortalised cell line is not an ideal model for primary cells. Not only were the BEAS-2B cells more mesenchymal than primary cells taken from healthy non-smokers, but they also demonstrated differences in how they responded both to stimulation with TGF-β or CSE and to the drug treatments used in this study. In particular, the molecular mRNA level differences between the immortalised and the primary cells suggested that BEAS-2B cells may not be good models for healthy bronchial epithelium.

In this study, TGF-β also exhibited lower levels of activity in BEAS-2B cells than expected from the literature, not managing to fully induce EMT in the immortalised cell line, nor in the primary cells taken from non-smokers. However, the cells trended towards an EMT-like

expression profile. Exposure to CSE failed to elicit a full EMT response in primary cells from non-smokers, although it did elicit a partial EMT-like phenotype without affecting the Smad signalling pathway. This further suggests that acute exposure to CSE is not sufficient to trigger the CSE-TGF-β-EMT pathway in healthy airway epithelial cells, although it may trigger other pathways to promote EMT-like changes.

Of the three drugs studied, neither salmeterol, tiotropium or fluticasone had any significant suppressive effect on EMT in either primary or BEAS-2B cells. Tiotropium, in particular, appeared to actually promote EMT in primary cells taken from non-smokers and smokers with normal lung function.

Overall, this study confirmed that primary bronchial epithelial cells can be successfully cultured and demonstrated that cryopreservation of these cells is possible, although smoking status had a negative impact on their viability. BEAS-2B cells did not appear to be a reliable model of healthy primary epithelial cells, particularly at the mRNA level of EMT-related expression. Cells from people with airflow limitation which did not reach the level of COPD did not appear to be undergoing active EMT, suggesting that EMT may only be active in fully developed COPD. Of the drugs used for treatment of COPD, only salmeterol indicating a trend towards suppression of EMT, while tiotropium and fluticasone appeared to have no suppressive effect on the process, with tiotropium promoting a partial EMT phenotype.

1. Introduction

1.1. Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is a progressive and irreversible limitation of airflow, caused predominantly by cigarette smoking in developed countries. As of December 2016, the Australian Institute of Health and Welfare (AIHW) reported that 5% of Australians over 45 have COPD, although this statistic is based on self-reporting. A cross-sectional study of the lung function of Australians aged over 40 reported that COPD affects 7% of all Australians over the age of 45 and 30% of those aged over 75 [1]. According to the World Health Organisation (WHO), COPD is currently the fourth leading cause of death worldwide, killing 3.17 million people per year in 2015 (Figure 1.1-1) and the AIHW placed it as the fifth leading cause of death in Australia in 2014, responsible for 4.6% of all deaths in Australia and killing 7,025 people. It has been estimated that COPD prevalence is grossly underestimated, with 45-65% of cases remaining undiagnosed, as patients believe their symptoms are part of the natural aging process [2]. Although COPD is caused mainly by smoking tobacco products in developed countries, exposure to biomass smoke from cooking-fires and the like also contributes in developing countries [3].

The main clinical features of COPD are shortness of breath, which often worsens upon exertion, and a chronic cough which may or may not be productive. For a diagnosis of COPD to be made, a patient undergoing spirometry must exhibit less than 70% predicted lung function, which is the ratio between the forced expiratory volume in one second and the forced vital capacity of the lung, and this must not be significantly improved following application of a bronchodilator [4]. As the disease progresses, patients also experience events

Useful abbreviations

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NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
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known as ‘acute exacerbations’ or, simply, exacerbations, which are sharp declines in lung function and health that necessitate a change in medication and management [5, 6]. COPD places a large burden on both the individual and the community.

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1.1.1. Burden of COPD

On an individual level, COPD can have enormous impact on the quality of life experienced by the affected person [7]. People may have difficulty breathing during light exercise or while doing mundane activities such as climbing stairs in earlier stages of disease. In later stages, as the disease worsens even simple activities such as getting dressed or showering can become difficult [8-10]. People experience less restful sleep, and overall emotional satisfaction and happiness decreases as the disease progresses[10]. The costs associated with treatment and living with the disease can also be prohibitive.

At the level of the community, the impact is mainly in the financial sector. Between 2011 and 2012 the average length of stay for hospitalisations resulting from chronic respiratory disease, including COPD, was 5 days [11] and according the AIHW, an average of 690,000 people over the age of 45 are hospitalised each year in Australia with COPD as the primary diagnosis (based on data from 2014-15). In 2011-2012, COPD was responsible for 398,000 hospital patient days, which accounted for67% of the total hospital days for all chronic lung diseases [11]. COPD accounted for 3.4% of the total disability-adjusted life years (DALYs), a measurement which is used to represent the burden of disease, in Australia in 2010 [11] .

Healthcare costs associated with COPD make it an extremely expensive chronic condition for a patient to have. In the UK a person with COPD spends up to £3,376 a year on treatment for the condition and related medical expenses, if adherent to combined long-acting beta-agonist and inhaled corticosteroid treatment [12]. Patients who are not adherent spend £2,373 a year on the disease; these values do not include monetary loss due to time off work and are purely expenses paid by the patient. In Finland in 2006 the direct and indirect cost of COPD totalled €107,705,570 [13] and in 2008 the economic cost of COPD in Australia was AU$8.8 billion

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according to a report commissioned by Lung Foundation Australia [14]. Despite this burden, there is no cure for COPD, and treatments can only slow disease progression, not halt it. This is in part due to the fact that the underlying molecular mechanisms and cellular processes of COPD still remain to be elucidated.

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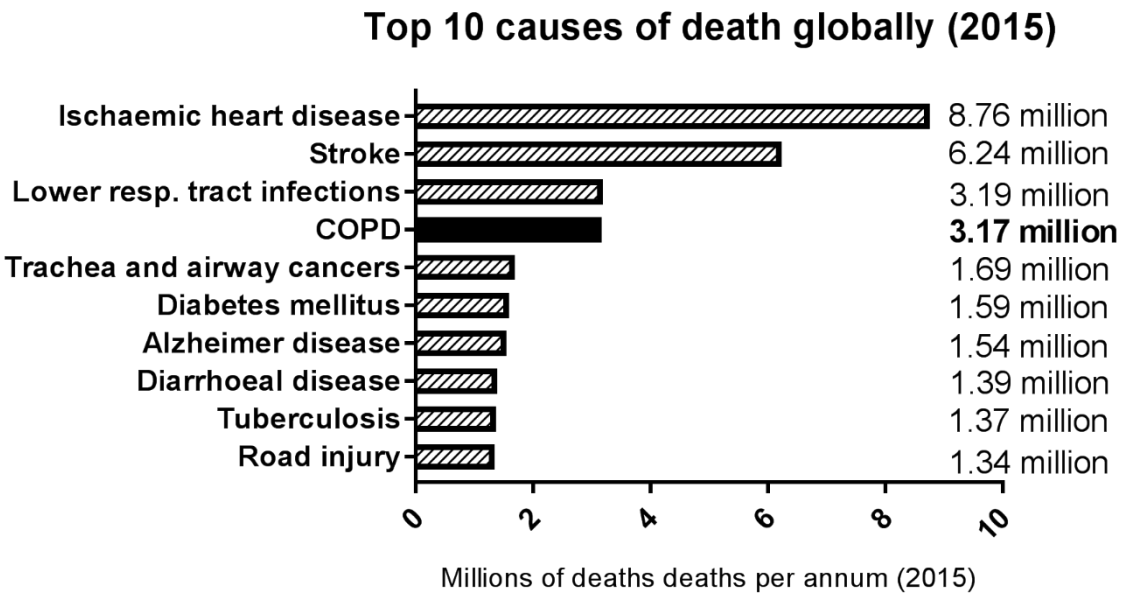

Figure 1.1-1: WHO data for the leading causes of death worldwide. *Top ten causes of death for 2015 are shown. COPD ranks fourth, with a total of 3.1 million deaths. It ranks below ischaemic heart disease (7.4 million) and stroke (6.7 million) and is only slightly overshadowed by lower respiratory tract infections, which may share some overlap with COPD, due to increased infection rates in COPD, as may the lung cancer statistics*
Figure created using data from WHO factsheet 310 (http://www.who.int/mediacentre/factsheets/fs310/en/).

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1.1.2. Pathology of COPD

Cigarette smoke exposure causes the majority of COPD cases in developed countries and smoking is the most important risk factor for the disease [4]. However, cigarette smoke's highly complex chemical composition means that the mechanisms which cause COPD are still not fully explained. The large airways are classically considered the major site for the occurrence of lung cancers in COPD, however with the increased prevalence of adenocarcinomas in recent years, the small airways are now beginning to become the major site of cancer in COPD [15, 16]. However, the large airways are still an important site in the disease pathology, hosting a number of pathological changes in COPD, including luminal inflammation, mucus hypersecretion, airway wall and epithelial thickening and epithelial-mesenchymal transition (EMT).

Classically, COPD is defined as chronic inflammation in the bronchi, or bronchitis, accompanied by fibrosis or narrowing of the airways, especially in the small airways [17], and in later stages by destruction of the parenchyma and alveoli, emphysema. Squamous cell cancer, mucus hypersecretion and chronic luminal immune infiltration are commonly seen in the large bronchi, those in the upper part of the respiratory tree which are supported by cartilaginous structures, while the small airways, those lower in the bronchial tree and without cartilage, suffer from fibrotic destruction, adenocarcimonas and loss of elastic recoil support provided by the parenchyma [17, 18].

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1.1.2.1. The healthy large airway

When discussing pathology, it is useful to briefly describe the normal physiology and structure of the airways. Given the focus of the project, this will be the large airways; the small airways differ in a number of features from the large, however they are not of major importance to the current discussion. In healthy large airways, as elsewhere, the epithelial layer is the body's primary defence against invading pathogens, foreign objects and noxious particles [19]. In the airways, it produces mucus which traps pathogens and particulates, and the cilia on the apical cell surface act to clear the mucus and invading bodies. The epithelium in healthy large airways is a pseudostratified layer, made up of ciliated epithelium and mucus producing goblet cells and glands [19]. However, due to its role as the first line of defence, the epithelium is exposed to any number of stimuli which can alter its growth, functionality and may render it malignant rather than protective.

1.1.1.1. *Epithelial layer dysregulation*

One of the key changes in COPD, and the main focus of this thesis, is the epithelial lining of the large airways. Additional to the potential for malignancy described below, in section 1.1.2.6, the airway epithelium in COPD exhibits squamous metaplasia, where in healthy lungs it forms a pseudostratified layer [20, 21]. As well as metaplasia, which causes epithelial thickening, the mucus producing goblet cells and sub-mucosal glands of the epithelium multiply and become over-active, producing thick mucus in the lumen which contributes to airway obstruction [22, 23], and there are also deficiencies in cilia length and function, which impede airway particulate clearance [24, 25]. Furthermore, there is evidence that the basal cells, from which the epithelial layer is renewed constantly, are dysregulated [26-30] and express mesenchymal markers such as S001A4, vimentin and α-smooth muscle actin [31-34]. This evidence suggests that EMT, which is the major focus of this thesis, may be active in COPD, and it has the potential to be a driver of fibrosis and the development of lung cancer in COPD [35], such as in the large airways, which are the main site of COPD-related squamous cell lung cancer [16].

1.1.2.2. Small airway destruction and emphysema

Loss and fibrosis of the small airways is strongly related to decline in lung function in COPD [17]. The exact mechanisms behind the remodelling and destruction of the small airways remains largely unclear. While inflammation in the small airways has been proposed to be major factors in airflow limitation [36], recent evidence has brought this into question, demonstrating hypocellularity of the small airway wall [37] and other mechanisms, such as EMT, are likely to play a role [38-40].

In addition to changes in the airways, emphysematous changes occur in the lung parenchyma of around 50-60% % of people with COPD [41]. Emphysema is destruction of the alveoli and surrounding parenchymal tissue of the lungs and while it also correlates strongly with worsened lung function [17], it is not the predominant cause of lung function decline in early stages of COPD. However, both emphysema and small airway changes fall beyond the scope of this study.

1.1.2.3. Infection

Respiratory infection rates in patients with COPD are increased compared to the general population [42]. Viral and bacterial infections are known to trigger exacerbations, with 30-55% of hospitalised exacerbations presenting with viral or bacterial infections [43-46]. However, the exact mechanism behind this relationship is unknown. Acute infections are not the only concern in COPD; around 40% of people with COPD are estimated to have chronic and persistent colonisation of the airways and lungs, which likely contribute to acute exacerbations [42]. H*aemophilus influenzae, Staphylococcus aureus, Streptococcus pneumoniae* and *Pseudomonas aeruginosa*, are all common bacterial pathogens in COPD, although *H. influenzae* appears more frequently than the others [42, 43, 47, 48], and once established the infections can be near-impossible to eradicate. The method by which these infections persist, and why these particular species are so overrepresented in COPD is currently unclear, although there is evidence that the ability of *H. influenzae* and *S. pneumoniae* to adhere to platelet-activating factor receptor (PAFr) on the airway epithelium may play a key role in their success [49-52], which is discussed in more detail in Chapterpter 7. While high rates of infection and persistent colonisation of the lungs by pathogens might indicate underactivity or suppression of the immune system, the longstanding view of the COPD lung is one of high levels of immune activation and infiltration and related chronic inflammation.

1.1.2.4. Inflammation

Inflammation has been classically thought to play a key role in COPD, with high levels of neutrophils, macrophages and other inflammatory cells seen infiltrating the airway walls, parenchyma, and airway lumen [53]. In particular, CD28 positive inflammatory cells and neutrophils are known to be upregulated in COPD and are thought to contribute to the commonly-observed glucocorticosteroid resistance seen in COPD [54-56]. Chronic inflammation has also been suggested to promote the development and growth of lung cancer [35, 57] and is known to promote the development of fibrosis in other diseases [58, 59]. However, the inflammation story is not clear-cut [60], as recent work has also demonstrated that the airway walls in COPD are actually hypocellular, which challenges this long-held view that inflammation in the airway wall is a major contributor to the airway wall pathology of COPD [37, 61] and suggests that there may be other key contributing factors to disease progression.

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1.1.2.5. Fibrosis

Fibrosis, airway remodelling and small airway obliteration are key features of COPD and have been shown to occur in areas with chronic inflammation in a number of other tissues, including the kidney and liver [58, 59]. It is proposed that in pathological fibrosis the normal cellular repair mechanisms are dysregulated and these healthy processes become damaging due to their continued activation [59]. Pro-inflammatory and pro-fibrotic cytokines such as tumour necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) are known to be unregulated in non-COPD related fibrosis [62-65]. In COPD airways, there are a number of similar cytokines upregulated, including TNF-α, IL-1, IL-8 and IL-10, [66] however it is still unclear precisely what effect they have in COPD. Possibilities include upregulation of extracellular matrix (ECM) production by existing fibroblasts or increased numbers of fibroblasts, which in turn results in higher levels of ECM production. Alterations at the cellular level in diseases such as these may open the door to more malignant changes, such as those seen in cancer.

1.1.2.6. Lung cancer

People with COPD have a six-fold increased risk of developing lung cancer [67, 68]. This risk is independent of their age, gender and current smoking status, which indicates that COPD itself is an independent predictor of lung cancer risk. In particular, while people with COPD are classically more prone to developing squamous cell and small-cell carcinomas than adenocarcinomas [69-71], there is evidence that adenocarcinomas are surpassing squamous cell carcinoma rates[16]. There is also evidence to suggest that adenocarcinomas are more common at GOLD Stage I, with later GOLD Stages being more prone to squamous cell carcinoma [72], and there is a difference in the rates of mortality due to lung cancer at these stages as well, with 55% of mild COPD deaths being due to lung cancer, while only 33% of severe-stage COPD deaths attributable to the same [16]. Both squamous and small-cell carcinomas occur most frequently in the central large airways [16]. The reasons behind this increased risk of lung cancer are largely unknown; it was thought to be due to the fact that the majority of people with COPD are smokers, however with the revelation that COPD predicts lungs cancer independently of smoking status, it would appear that some mechanism involved in COPD pathogenesis promotes or even causes the development of lung cancer. Along these lines, it has been proposed that susceptibility to COPD is related to susceptibility to lung cancer [73]. Metastatic cancers, including lung cancers, are shown to undergo a process called epithelial-mesenchymal transition (EMT) [74] and according to Cancer Research UK and the National Institute of Health (NIH) Surveillance, Epidemiology and End Results fact sheets, epithelial cancers, or carcinomas, comprise 85-99% of all human cancers [75, 76], which suggests that the epithelium is particularly susceptible to malignant changes.

1.2. Epithelial-mesenchymal transition

1.2.1. What is EMT?

Epithelial-mesenchymal transition (EMT) is a process that occurs naturally as part of human development. There are three 'types' of EMT, which occur at different points in development or in response to different stimuli (Figure 1.2-1). During embryogenesis, Type 1 EMT causes the formation of the mesodermal ridge and allows the original embryonic epithelial cells to differentiate into the many types of mesenchymal structures in the body [77, 78]. However, EMT occurs even after development is complete; Type 2 EMT is involved in wound-healing and repair. It allows epithelial cells to migrate to the site of injury and close wounds, as well as promoting the formation of scar tissue via fibrosis. Both Type 1 and Type 2 EMT are normal and not pathogenic.

However, Type 3 EMT is associated with a number of pathogenic processes. Similar to Type 2 EMT but made distinct by the presence of hypervascularity, Type 3 EMT is associated with cancer metastasis [35] and a number of fibrotic diseases [65, 79-82] and is the major focus of this project.

EMT occurs when the epithelial layer loses coherence and becomes disorganised. Cell-to-cell contacts which use E-cadherin are replaced by N-cadherin junctions, and other adherent molecules such as occludins and caludins are downregulated [83]. Additionally, the cells undergo remodelling of their cytoskeletal structures, losing cytokeratin and upregulating expression of mesenchymal proteins such as vimentin, α-smooth muscle-actin and S100A4. The cells gain motility and lose their ability to form the tight barrier layer characteristic of epithelial cells. They also change their structure, losing apical-basal polarity and instead

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gaining an elongated, motile phenotype. Cells undergoing EMT may also begin to produce extracellular matrix remodelling proteins such as fibronectin, matrix-metalloproteases and overall become functionally more similar to mesenchymal cells such as myofibroblasts.

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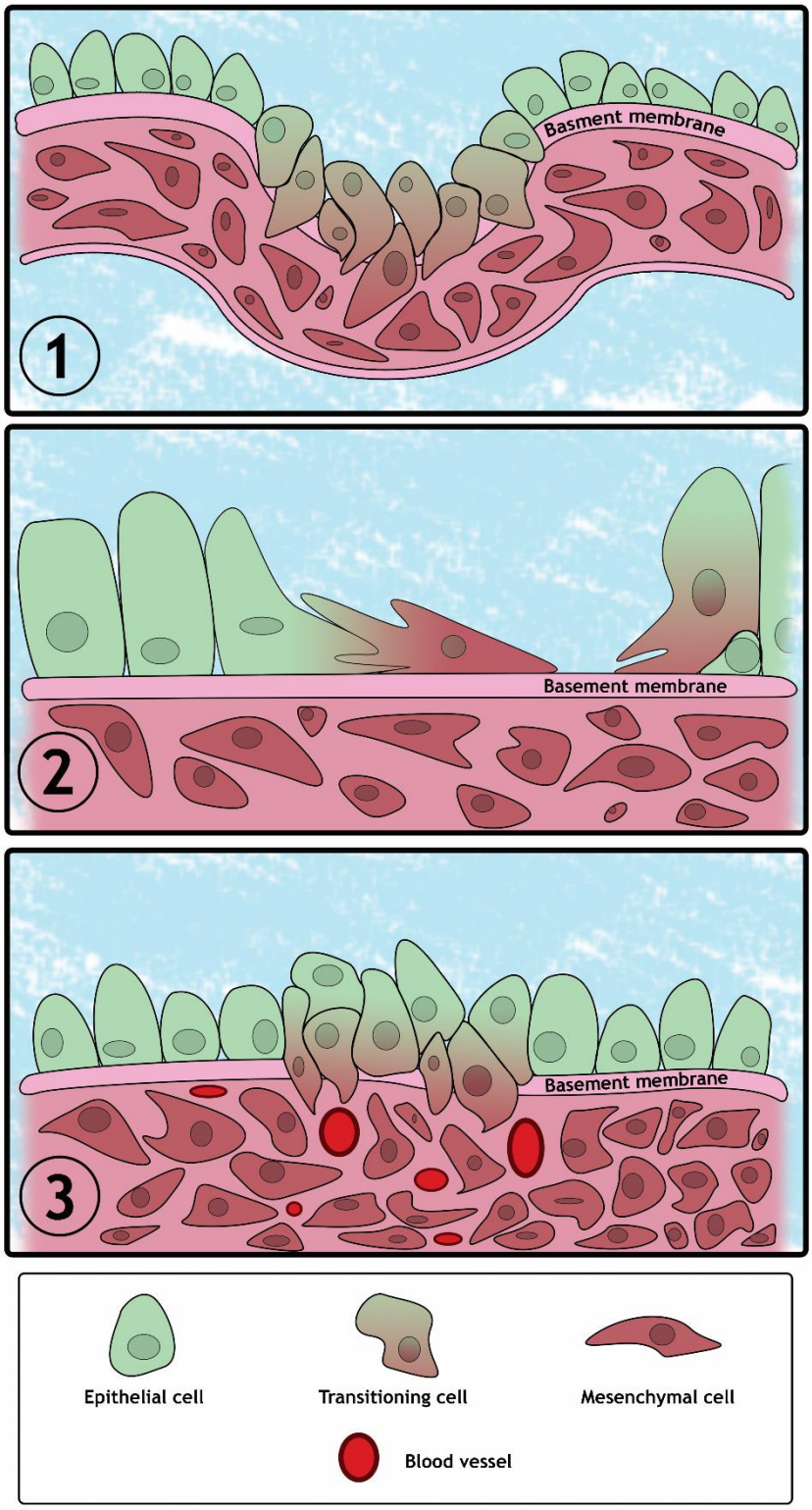

Figure 1.2-1: The three types of epithelial-mesenchymal transition (EMT). *Type 1 EMT occurs during embryogenesis and is responsible for forming the mesodermal ridge and the later formation of the mesodermally-derived tissues and organs. Type 2 EMT is active in adult tissues and allows for wound healing and repair of damaged epithelial layers via migration of new cells. Type 3 EMT is associated with malignant and pathogenic processes such as metastasis in cancer and fibrotic diseases. Type 3 EMT is the only one of the three types of EMT which is associated with hypervascularity in the area.*

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1.2.2. What causes EMT?

EMT can be driven by a number of different processes, and within disease its precipitating factors are often unclear, and are likely to be multifaceted. The intracellular processes by which EMT occurs appear to be centred around the activation and regulation of the transcription factors Snail and Slug, TWIST and zinc-finger E-box binding transcription factors [83]. These pathways are not unique to EMT; they control a number of other vital processes and can be triggered by a number of different factors, both internal and external to the cell. Thus, while these pathways are clearly indicated as involved in EMT development and progression, the triggers within active disease are harder to unravel.

1.2.2.1. The role of TGF-β in EMT

One of the most widely-studied inducers of EMT, both *in vivo* and *in vitro*, is transforming growth factor-β1 (TGF-β) [83]. TGF-β has shown to be linked to EMT in hepatic fibrosis [65, 80], kidney fibrosis [79, 81] and non-COPD-related pulmonary fibrosis [82], in addition to endothelial-mesenchymal transition in cardiac fibrosis [84]. It is also known to be upregulated in the small airways of people with COPD [85, 86]. TGF-β acts on TGF-β receptors 1 and 2 to form a complex. This complex then activates the Smad2/3 complex via phosphorylation, and the phosphorylated Smad2/3 can then either bind Smad4 and move into the nucleus to act as a transcription factor, or Smad6 or Smad7 can bind the complex and prevent transcription [87-89] (Figure 1.2-2). The Smad pathway is the most extensively studied of the pathways through which TGF-β acts, although there is evidence to suggest that the MAPK pathway, which can be activated by exposure to TGF-β and also to cigarette smoke, may play a role in inflammation in COPD [90]. Even under *in vitro* conditions TGF-β can induce EMT in bronchial epithelial cells [33, 91].

One of the transcription factors which the Smad pathway regulates via the MAPK pathway [92] and which is capable of promoting EMT, along with the Snail/Slug and ZEB families, is TWIST [93]. TWIST is a helix-loop-helix transcription factor which has been demonstrated to promote EMT in metastatic cancer [94]. It is not expressed commonly in healthy adult tissues [95], and has been proposed as an attractive target for potential pathway blocking therapeutics in breast cancer [96]. TWIST is an e-box binding transcription factor and is naturally involved in embryonic development and differentiation, but in adult tissues promotes EMT via the down-regulation of E-cadherin [92, 95].

Factors other than TGF-β can induce EMT, although TGF-β is considered to be a major contributor to pathological EMT. TNF-α induces EMT in bronchial epithelial cells, and can enhance the EMT-inducing effects of TGF-β in-vitro [91]. EMT can also be induced by estradiol and the surface glycoprotein CD44 in breast cancer, which shows that drivers of EMT can be tissue-origin specific [97, 98]. EMT, whether induced by TGF-β or not, plays not only a role in development but is also involved in a number of disease pathologies.

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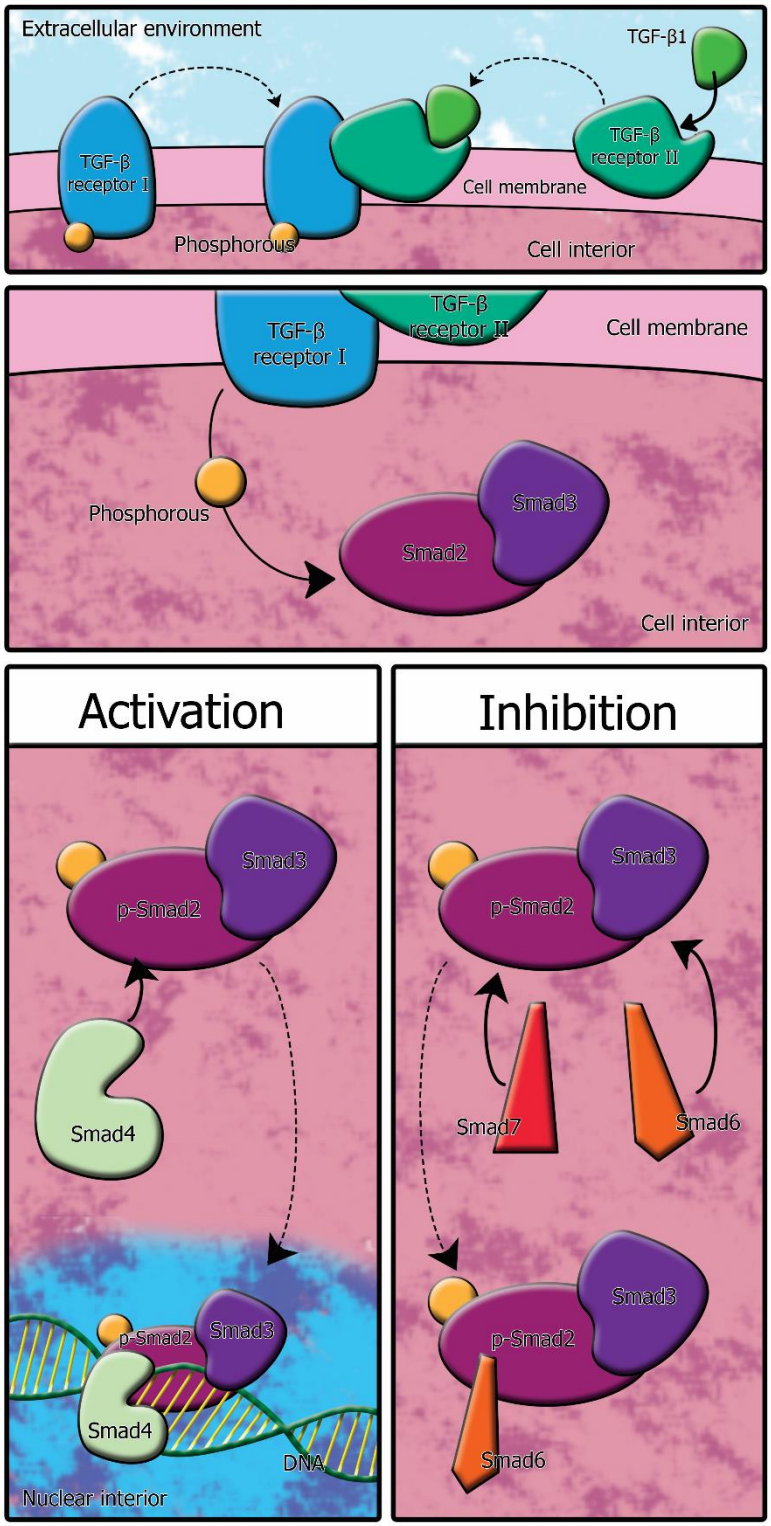

Figure 1.2-2: Transforming growth factor-β1 (TGF-β) activation of the Smad pathway. *TGF-β binds to TGF-β receptor II (top panel). Following the binding of TGF-β, TGF-β receptor II dimerises with TGF-β receptor I. Once the dimer is formed, TGF-β receptor I phosphorylates the Smad2/3 complex (middle panel). If the Smad signalling pathway activates successfully (bottom-left panel) then Smad4 binds the phosphorylated Smad2/3 complex and the active complex translocates to the nucleus, where it acts as a transcription factor. If the Smad signalling pathway is inhibited, instead of Smad4 binding the phosphorylated Smad2/3 complex, either Smad6 or Smad7 bind and inactivate the complex (bottom-right panel).*

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

1.2.3. EMT in COPD

While EMT is especially well-recognised to occur in many forms of metastatic cancer, including lung, gastric and breast cancers [99-102] and is also known to occur prior to kidney, hepatic and pulmonary fibrosis [65, 79-82], the potential for its presence in the development of COPD was not considered until relatively recently. In 2010 Sohal and colleagues noticed fragmentation of the reticular basement membrane in the airways of people with COPD [31]. Along with this fragmentation, they noted cells appeared within these clefts; however the reticular basement membrane is normally acellular. These cells stained positive for the epithelial cell marker cytokeratin 5, suggesting that they may have originated in the epithelial layer. However, they also stained positive for mesenchymal cell markers, including S100A4 and matrix metalloproteinase-9 (MMP9), as did some cells in the epithelial layer. Based on these observations, Sohal and colleagues proposed that EMT may be occurring in the airways of people with COPD and provided further support for these findings with additional cellular counting of immunohistochemically stained tissue in 2011 [32].

These findings were later corroborated by Milara and colleagues in 2013, who demonstrated that primary human bronchial epithelial cells (pHBECs) underwent EMT in response to exposure to cigarette smoke extract (CSE) in both submerged and air-liquid interface (ALI) cultures, in addition to exhibiting decreased cell-to-cell contact as demonstrated bydecreased trans-membrane electrical conductance [33]. Milara and colleagues confirmed that the EMT pathway in pHBECs appeared to be at least partially Smad dependant, and that cigarette smoke exposure induced these cells to release increased levels of TGF-β and undergo EMT. They also demonstrated that the phosphodiesterase 4 (PDE4) inhibitor roflumilast-N-oxide

can prevent EMT in response to cigarette smoke, suggesting a potential treatment and an alternative non-TGF-β reliant mechanism of action of EMT in COPD [103].

More recently, Nishioka and colleagues have shown that soluble factors secreted by fibroblasts in COPD can induce EMT in healthy epithelial cells. While they did not identify the specific factors responsible, these findings provide further weight to the theory that EMT may occur in COPD airways, and raise the need for further study of the process in COPD [34].

Overall there is a clear picture emerging that EMT is occurring in COPD and in direct response to cigarette smoke exposure. The precise mechanism by which the process is activated remains unclear, and it is highly likely that there are a number of mechanisms that trigger its progression, including Smad [33], PPAR [104-106], cholinergic [107-110] and Wnt pathway signalling [104, 111, 112]. Further research into the role of EMT in COPD is needed, in addition to uncovering the pathways which activate it in the disease.

Useful abbreviations

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1.2.3.1. EMT in lung cancer

As mentioned previously, people with COPD have a six-fold increased risk of developing lung cancer [67, 68]. The mechanism behind this increased prevalence is currently unknown, however the discovery of EMT in COPD and smokers' airways may shed light on the phenomenon. EMT is one of the key processes involved in cancer development and malignancy, and 85-99% percent of all cancers are derived from the epithelium [75, 76], which suggests a particular susceptibility to malignancy. In particular EMT is strongly linked to the metastatic potential of a number of different cancers, including ovarian [113], breast [101], lung [114] and prostate [115, 116]. The process as it occurs in lung cancer is of particular relevance to COPD and the relationship between the two diseases. Lung cancers exhibit EMT during metastasis and a tumour's ability to metastasise and invade surrounding tissues correlated with the degree of EMT [102, 117].

EMT in lung cancer is thought to be driven at least in part by TGF-β [118-122] and cytokines such as interleukin-17 [123], acetylcholine [124] and Williams syndrome transcription factor [114]. An inflammatory microenvironment in the airways has been linked to the development of cancer, supporting the role of inflammatory cytokines as potential drivers of EMT [35, 57, 125]. However it is not known if these same molecules drive EMT in COPD.

1.3. Modelling human disease and mechanisms *in vivo* and *in vitro*

When studying disease, it can often be challenging to look at the process in patients. This may be due to any number of reasons, including the rarity or lethality of the disease, the invasiveness of the procedures required to study the affected organs or the inability to detect the early stages of disease progression, which limits the ability to study and preventdisease development. In these cases, it is desirable to model the disease or process and utilise the model to identify key pathways which can become the target of therapy. There are two types of model systems available in the study of disease: *in vivo* and *in vitro* models. *In vivo* models of respiratory disease typically utilise mice, rats, guinea pigs or, rarely, dogs [126-128]. These models are useful for examining the complex intra-cellular interactions seen in human disease, however they are costly and time-consuming to produce and often do not recapitulate the precise pathology seen in human disease.

In vitro cell-based models have the advantage over animal models in that they are sourced directly from human tissue and thus are more likely to be accurate representations of the human condition (Figure 1.3-1). However culture-based models lack complexity and intracellular interactions, which makes them less relatable to the human condition as a whole. While there are some culture models which allow multiple cell types to be grown concurrently [34, 129-131], they still only model a fraction of the complexity of the human body. Additionally, culture conditions can impact cell phenotypes, and there are marked differences between commercially available immortalised cell lines and cells taken directly from patients with the disease of interest [132]. Immortalised and primary cells have different advantages and disadvantages in the study of disease, (Figure 1.3-2) and it is important to

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CSE *– cigarette smoke extract*

consider these when choosing a model. Despite their drawbacks, both *in vitro* and *in vivo* models can shed light on different aspects of the disease of interest.

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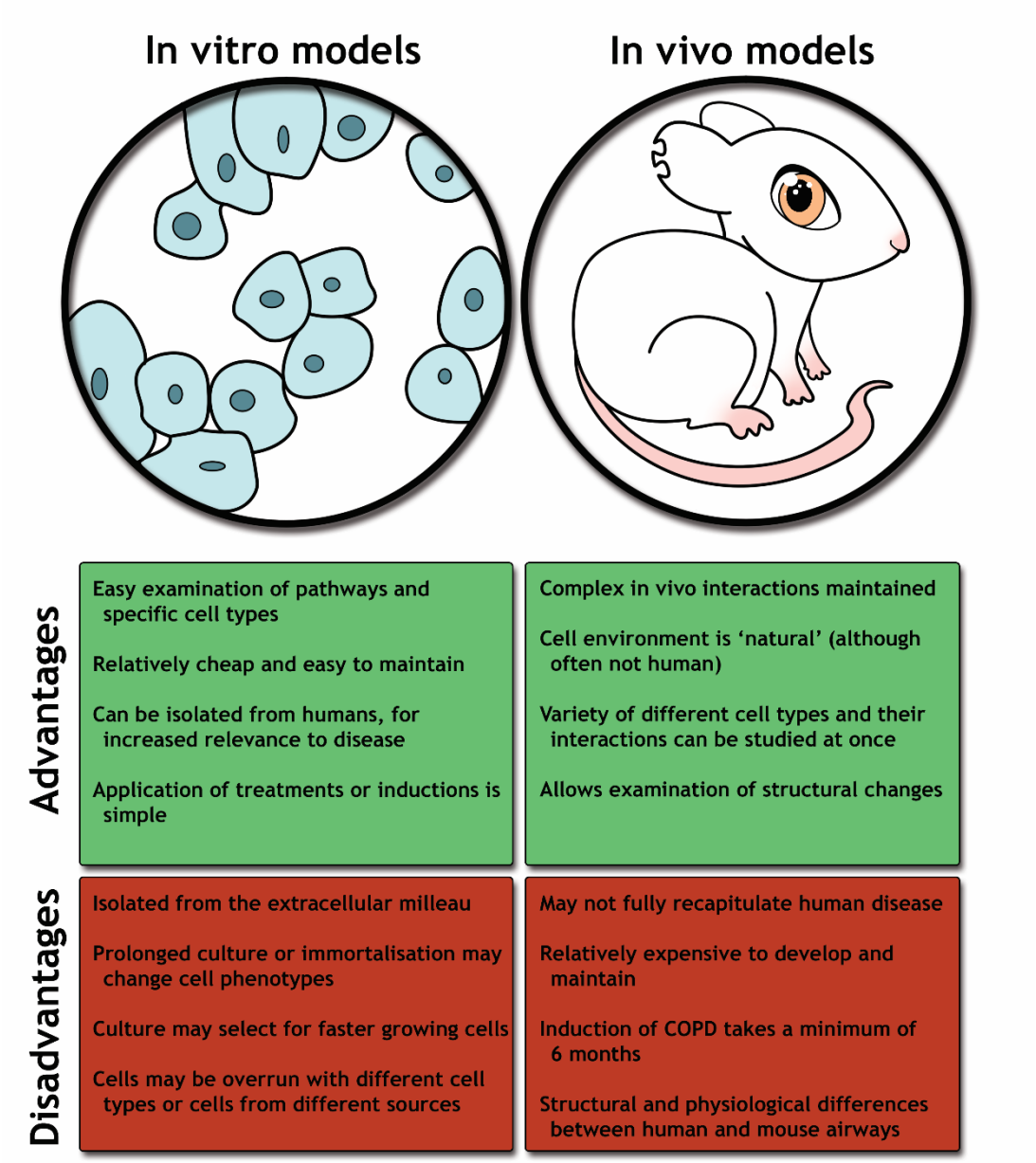

Figure 1.3-1: Comparison of advantages and disadvantages of in vitro models versus in vivo animal models. *Both cell-based models and models of disease induced in animals have advantages and disadvantages. Cell models (left) can be isolated from humans and specifically from diseased individuals, however lack the complex interactions of the extracellular milieu and do not provide structural information. In contrast, animal models allow for examination of histology and systemic effects, however structural differences between the model and humans are an issue.*

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1.3.1. Current models of COPD

1.3.1.1. In vivo models

Briefly, *in vitro* models of COPD are commonly induced in rats and mice, with a number of guinea pig models also being used [133-135]. There are three main methods of induction, these being genetic, exposure to exogenous elastase and exposure to cigarette smoke or other airway irritants. Genetic models of COPD [136-138] and elastase induced models [43, 139-143] typically more closely resemble human alpha-1-antitrypisn (AAT) deficiency caused COPD, which is distinct from smoke induced COPD [144-146]. Even in cigarette smoke induced rodent models [133, 136, 140, 147-154], the endpoint which is typically used as the measure of COPD induction is emphysema [126]. This is problematic, since emphysema only occurs in half of all human COPD cases, and is a late-stage development [41].

Additionally, structural differences between human and rodent airways [127, 133, 134] mean that key COPD indicators in humans such as mucus cell hyperplasia and fibrosis are often not measured. Small airway changes, which are key to loss of lung function in humans [36] are also difficult to measure, as mice lungs do not possess terminal bronchioles, and the closest analogous airway structure to the large airways in humans is actually the trachea, making physiological comparisons challenging [135]. Despite these drawbacks, rodent models of COPD have furthered understanding of COPD drivers, indicating the potential roles of pathways including PDGFR signalling via Sestrin-2 [136], angiotensin receptor signalling [151] and GSK-3 activation [155]. However, to understand complex, human-specific diseases such as COPD, it is often necessary to study human tissue and cells.

1.3.1.2. BEAS-2B and other immortalised cells

Immortal cell lines derived from people with COPD are not available commercially, and even commercially available airway and lung derived epithelial cell lines tend to have limited data on the health status of the donors. The most common models of airway epithelium are the A549 cell line, derived from cancerous alveoli epithelia, the 16HBE cell line, derived from human bronchial epithelia and the BEAS-2B cell line, derived from healthy human bronchial epithelia. None of these cell lines are taken from patients with COPD, and given the extent of their culturing it is likely that even if they had been, they would not accurately represent the *in vivo* condition [132]. However, they are useful for studying specific pathways that may be present in airway epithelium and active in COPD.

The BEAS-2B cell line is of particular relevance to this thesis. Regarded as 'normal', this cells line is an SV-40 adenovirus modified cell line taken from healthy human bronchi [156]. When studying airway epithelium, in the absence of primary cells the BEAS-2B cell line is considered an acceptable alternative, and its use in research has been and still is widespread [90, 157-177]. However, BEAS-2B cells clearly have an altered phenotype, evidenced by their immortality, their inability to differentiate into a fully formed epithelium when grown at an air-liquid interface [168] and their tendency to terminally differentiate when exposed to serum [178]. These characteristics raise the question: what else about their phenotype is different from primary epithelial cells extracted from healthy airways? With regards to EMT the two cell types, BEAS-2B and primary cells, have never been directly compared, since the immortalised cell line is widely accepted as a reference for 'normal'.

1.3.1.3. Primary cells

While immortalised cell lines can shed light on diseases, particularly if there is a specific triggering element or condition which can be applied to the cells, in cases such as COPD where the exact mechanisms and causes are unclear, primary cells taken from the airway epithelium of patients with COPD theoretically provide a more complete insight into the disease. While COPD is caused mainly by cigarette smoke in developed countries such as Australia and America, the exact components which trigger the pathological processes are unknown, as are the factors within the affected person which cause them to be susceptible. Indeed, even the mechanisms by which the disease develops and progresses remain largely unclear, although there have been large advances made in recent years, and it is becoming obvious that there are multiple cell types which play different roles in the disease. Since it is therefore difficult to induce an accurate model of COPD in a healthy cell line, particularly given the contribution of numerous different cell types to the disease, when studying bronchial epithelial cells and their role in COPD it may be simpler to take cells from people with COPD to study rather than attempting to artificially induce the full disease state in healthy cells. However, it is completely possible to induce certain mechanisms and changes which may drive disease progression, such as EMT, in healthy cells and thus it is possible to examine particular mechanisms behind diseases, albeit without the complete context of the disease itself.

Work has been done on a number of different cell types in COPD, including lymphocytes [54, 179], T-cells [55, 180-182], macrophages and natural killer cells [56, 183-188], lung-derived fibroblasts [189, 190] and, most importantly for this thesis, epithelial cells [25, 103, 189, 191-196]. Primary lung epithelial cells can be grown in submerged culture [103, 185,

193, 196], however for further applicability to the lung environment, they can also be grown in an air-liquid interface (ALI) culture system, which allows them to form a differentiated epithelial layer with cilia and mucus production [103, 189, 193-195]. There has been evidence that epithelial cells derived from people with COPD do not survive as well as those from non-smokers or people without COPD, nor do they differentiate as readily at ALI as healthy epithelial cells [30], which can make using them as a model difficult. There is evidence that the major site of dysregulation occurs in the basal stem cells of the epithelial layer [26-30, 135, 197, 198], suggesting that submerged culture, which produces similarly 'basal' cells, can be exceptionally useful in studying these mechanisms.

Despite the challenges, epithelial cell culture models of COPD have revealed a number of insights into the disease. Smoking and COPD decrease the length of the airway cilia, possibly reducing clearance of bacteria and damaging particles from the lungs [25]. Heterogeneity of CFTR, an ion channel whose dysfunction is responsible for cystic fibrosis and which was speculated to contribute to chronic bronchitis in COPD, does not appear to impact ion transportation in COPD more than in non-COPD patients [195], and although the work was not performed in COPD-derived cells, there is evidence that exposure to cigarette smoke promotes autophagy in airway epithelial cells, which may play a role in COPD [194]. There is also evidence from cell culture that platelet-activating factor and the PI3K-p110α pathway may play roles in the increased rates of bacterial and viral infections seen in COPD lungs [191, 199]. However, the models which are most relevant to this thesis are the models which examine EMT with relation to COPD.

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
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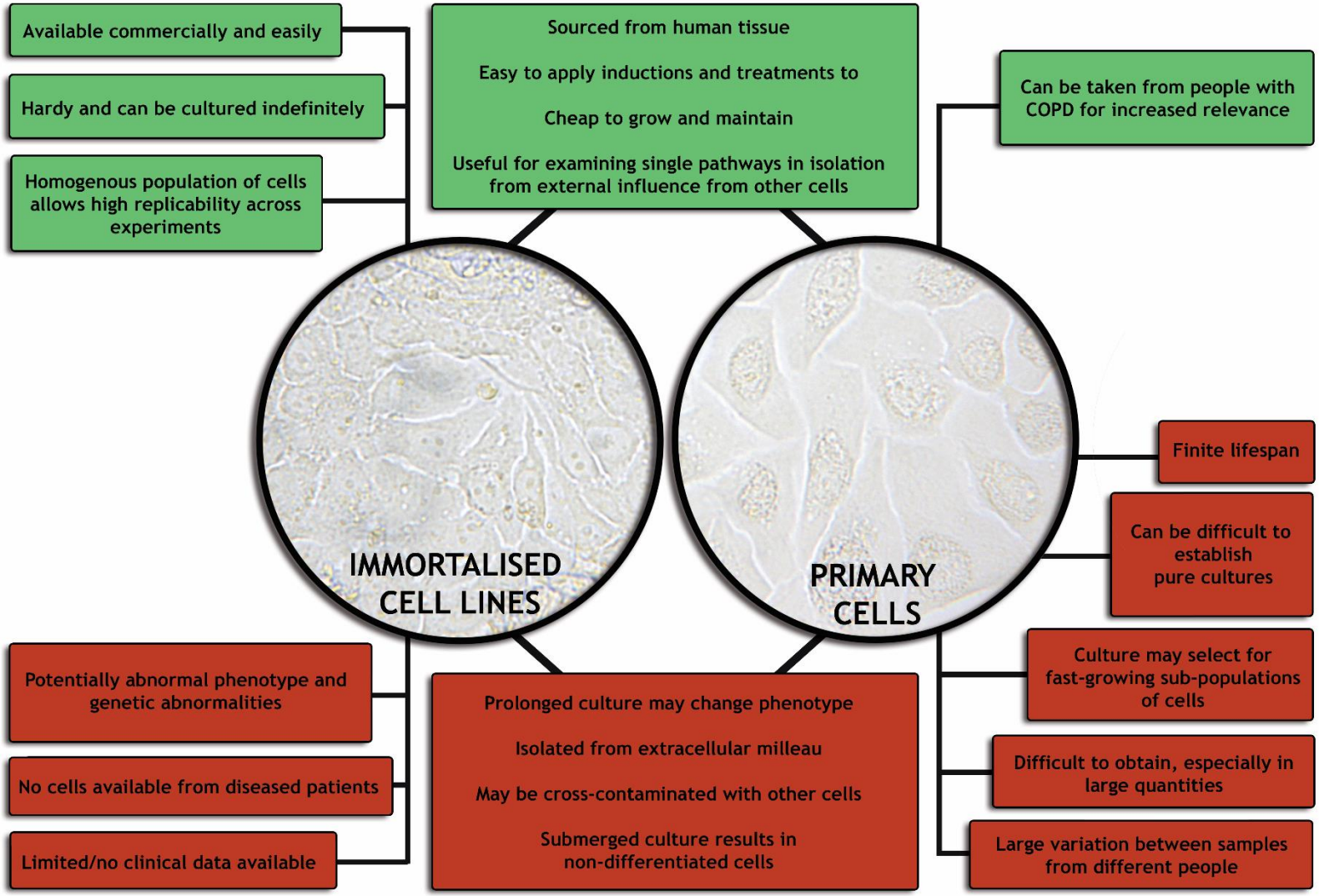

Figure 1.3-2: Comparison of the advantages and disadvantages of immortalised cell versus primary cell models systems. *In vitro models are valuable for studying pathways and mechanisms, as well as testing potential therapies. Immortalised cell lines are easier to obtain and culture than primary cells, however often exhibit abnormal phenotypes compared to primary cells.*

1.3.2. Current models of EMT

1.3.2.1. In healthy human bronchial epithelial cells

As has been shown in a number of other epithelial cell lines derived from various origins, EMT can be induced by application of exogenous TGF-β [62, 200, 201]. It comes as little surprise, in that case, that human epithelial cells derived from the alveoli (the A549 cell line) and the bronchi (the BEAS-2B cell line) undergo EMT in response to stimulation with TGF-β [163, 165, 175, 202]. Perhaps of more obvious relevance to the question of EMT in COPD, it has been demonstrated that nicotine can directly induce EMT when applied to cells, and appears to act through the Wnt/β-catenin signalling pathway [112]. However, as discussed previously (1.3.1.2), immortalised cell lines exhibit numerous changes from their original phenotypes, and thus it is important to know if these changes also occur in primary cells derived directly from human lungs.

Primary human bronchial cells (pHBECs) are epithelial cells taken directly from the airway walls via brushings during bronchoscopy, or grown out from biopsy samples. They have finite lifespans and are more difficult to obtain than commercially available cell lines, however they are theoretically more closely representative of the physiology of their origin than immortalised cell lines. While cell lines are useful, it is important to know if pHBECs are capable of undergoing EMT before making firm claims regarding the potential role of EMT and pathways which induce it in COPD based on cell culture work. EMT can be induced in primary human bronchial epithelial cells via application of TGF-β and tumour necrosis factor-alpha (TNF-α) separately, and there is a synergistic effect when the two are applied in combination [91]. Furthermore, application of cigarette smoke extract (CSE) not

only induces EMT in pHBECs, it also appears to activate the Smad pathway, which is one of the major TGF-β related pathways [33], and is capable of inducing EMT in alveolar-derived primary epithelial cells [203]. This suggests that TGF-β and its associated pathways may play an important role in COPD, however without evidence from diseased cells or tissue, this remains speculative.

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1.3.2.2. In COPD-affected bronchial epithelial cells

There have been no animal models to date which have looked for EMT changes in COPD airways, however there have been a number of human-derived *in vitro* cell models. Although EMT can be induced in epithelial cells derived from the airways and alveoli, as discussed previously, it is all for nothing if it does not mimic the EMT seen in COPD. While biopsies from patients can reveal some mechanistic information, they do not readily allow the testing of different treatments or the inhibition of pathways, which allows one to mine further information about the process of interest, although it can be done [204]. Cell culture allows these things to be done more easily, and the most reliable way to get the most accurate culture models of EMT in COPD is to culture epithelial cells directly from the airways of people with well-documented disease.

As early as 2006, it was suggested that there were some issues with epithelial cell deregulation and altered functioning that may be playing a role in COPD, beyond simple hyperplasia [205], however it was not until 2010 that evidence was provided for the possibility of EMT in COPD airways [31]. Despite being able to induce EMT in bronchial epithelial cells via the application of endogenous cytokines, growth factors and other stimulants such as cigarette smoke extract [77, 78, 82, 91, 163, 165, 202, 206], it was only in 2010 that Milara and colleagues demonstrated EMT in unstimulated cultured cells taken directly from people with COPD [33]. Since then, there have been a number of models of EMT in bronchial epithelial cells used to study EMT in COPD, both in primary cells derived from COPD and immortalised cell lines induced to exhibit EMT [34, 125, 197, 203, 207]. However there appears to have been little work done to answer a very basic question: in the absence of primary cells taken from people

with COPD, can immortalised cells truly recapitulate EMT as seen in COPD, or are they modelling a process which may appear superficially similar but where the underlying mechanisms are fundamentally different? There is also the question of whether EMT occurs during early, pre-clinical COPD or if it becomes active only once the disease is established.

Useful abbreviations

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COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
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CSE *– cigarette smoke extract*

1.4. Aims

1. To determine if EMT is present in primary bronchial epithelial cells sourced from non-smokers, smokers with normal lung function and people with airflow limitation or pre-COPD.
    - To collect and characterise a bio-bank of primary bronchial epithelial cells (pHBECs) taken from healthy non-smokers, smokers with normal lung function and people with airflow limitation, with a focus on EMT markers and the pathogen adhesion molecules I-CAM-1 and platelet activating factor receptor (PAFr).
    - To compare the expression of EMT-related proteins and mRNA in cultured pHBECs with the expression of the same markers in matched biopsy tissue in order to examine the effects of culture on the cells' expression profiles.
    - To compare the expression profiles of pHBECs from people with normal lung function and pre-COPD with regards to the expression of the lung cancer marker *brachyury* to determine if pre-COPD-derived epithelial cells exhibit increased expression of this lung cancer marker.
2. To assess the suitability of the BEAS-2B cell line as a model for pHBECs, with particular focus on EMT.
3. To induce EMT in pHBECs and immortalised cells via application of cigarette smoke extract and transforming growth factor-β, and determine whether cigarette smoke extract induced EMT occurs in healthy epithelial cells taken from non-smokers.

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4. To assess the effect of current late-stage COPD treatment drugs, including corticosteroids, long-acting muscarinic-antagonists and long-acting β-agonists on EMT in early pre-clinical COPD.
    - To determine if the drugs have similar effects on induced EMT as on EMT in cells isolated from people with airflow limitation.

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2. Materials and Methods

NOTE: Unless otherwise specified, procedures were performed under aseptic conditions in the PC2 laboratory at the Menzies Research Institute. All cell culture was performed in an appropriately sterilised laminar flow biosafety cabinet which was exposed to UV radiation for a minimum of 15 minutes prior to and following use.

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CSE *– cigarette smoke extract*

2.1. List of Abbreviations

2.1.1. General abbreviations

COPD – chronic obstructive pulmonary disease

CS – current smoker

CSE – cigarette smoke extract

ELISA – enzyme linked immunoabsorbance assay

ES – ex-smoker

FIJI – FIJI is just ImageJ

GOLD – Global Initiative for Chronic Obstructive Lung Disease

ICS – inhaled corticosteroid

IL - interleukin

LABA – long acting beta agonist

LAMA – long acting muscarinic antagonist

NLFS – smoker with normal lung function

NNS – non-smoker

p# – passage number

PAFr – platelet activating factor receptor

PBFs – primary bronchial fibroblasts

pHBECs – primary human bronchial epithelial cells

SEM – standard error of the mean

TGF-β – transforming growth factor beta-1

TJP1 – tight junction protein-1

TNF-α – tumour necrosis factor-alpha

2.1.2. Abbreviations for materials and methods

AmpB – amphotericin B

BEBM – bronchial epithelial basal medium

BEGM – bronchial epithelial growth medium

BSA – bovine serum albumin

DAB – 3'3'-diaminobenzadine

DMEM – Dulbecco's modified Eagle medium

DMSO – dimethyl sulphoxide

EDTA – ethylene diamine tetra-acetic acid

ECS – eight-chambered slide

FBS – foetal bovine serum

FEF_{25-75} - forced expiratory flow 25-75%

FEV_1 – forced expiratory volume in 1 second

FVC – forced vital capacity

HRP – horseradish peroxidase

PBS – phosphate buffered saline

penstrep – penicillin/streptomycin

PVP – polyvinyl pyrrolidone

qPCR – quantitative real-time PCR

RHH – Royal Hobart Hospital

WP – well plate

2.2. Recruitment and subject demographics

2.2.1. Recruitment methods

Recruitment and collection of samples was approved by the Tasmanian Health and Medical Human Research Ethics Committee (H0013051).

Volunteers who responded to a newspaper advertisement for the study were recruited and screened by a practice nurse, before attending the Royal Hobart Hospital (RHH) for a bronchoscopy. Volunteers were sedated by a trained anaesthesiologist and local anaesthetic applied to the nasal passages prior to insertion of the bronchoscope. All procedures were performed by fully trained staff within the RHH. The bronchoscope was inserted via a nostril, travelling down through the larynx and into the upper bronchi. Excess mucus and blood was cleared by flushing the airways with a sterile saline solution. Brushings were taken from the large airways and placed in bronchial epithelial growth media (BEGM) for culture into bronchial epithelial cells. Following the brushings, a small clamp was inserted into the airways. Four biopsies were removed and placed in 4% paraformaldehyde for embedding in wax blocks, and a single biopsy was removed and placed in Dulbecco's modified Eagle media (DMEM) supplemented with 10% FBS for use in cell culture.

2.2.2. Volunteer demographics

The volunteers who participated in this study responded to an advertisement requesting smokers and non-smokers. They were recruited and assigned to the study groups on the basis of airflow obstruction and smoking status. It was expected, based on prior experience by the group in which this study was undertaken, that there would be enough volunteers with COPD as defined by the GOLD criteria [208]. However, while the volunteers presented with airflow obstruction, all but two failed to meet the clinical criteria for a diagnosis of COPD under the GOLD guidelines (FEV_1/FVC ratio <70%) [208]. However, there was evidence of significant airflow limitation and obstruction in a subset of 17 volunteers (Table 2.2-1). These volunteers exhibited significantly lower forced expiratory volumes over one second (FEV_1), as well as reduced maximum mid-expiratory flow (also called forced expiratory flow 25-75% or $FEF_{25-75\%}$) and reduced FEV_1/FVC ratios as compared to those volunteers with normal lung function, as determined by ANOVA. For the purposes of this study, these patients were considered to have chronic airflow limitation, or 'pre-COPD', as they exhibited significant airflow limitation, and are referred to as 'COPD' for the remainder of this work.

Overall, this study successfully recruited 46 volunteers out of 117 people who responded to the advertisement (Figure 2.2-1). In addition, four volunteers who were already undergoing a bronchoscopy for medical reasons were recruited at a later stage in the study. The final breakdown of volunteers was 19 non-smokers, 12 ex-smokers with chronic airflow limitation/COPD, 5 current smokers with chronic airflow limitation/COPD and 10 current smokers with normal lung function.

Table 2.2-1: Demographic information for the volunteer groups which provided samples. *Data were presented in the format: mean (standard deviation), except for gender, which was presented as total females/total males. One pack-year is equivalent to smoking 1 pack (20 cigarettes) per day for 1 year. Numbers in* ***bold*** *are significantly different from non-smokers (one-way ANOVA p<0.05), numbers preceded by an asterisk (*) are significantly different from smokers with normal lung function (one-way ANOVA p<0.05), numbers preceded by a cross (†) are significantly different from current smokers with COPD (one-way ANOVA p<0.05).*

KEY: NNS= non-smokers; NLFS=smokers with normal lung function; COPD= chronic obstructive pulmonary disease; COPD-CS= current smokers with COPD; COPD-ES= ex-smokers with COPD; FEV_1= forced expiratory volume in 1 second; FVC=forced vital capacity; FEF=forced expiratory flow.

Group	*Gender (female/male)*	*Age (years)*	*%Predicted FEV_1*	*%Predicted FVC*	*FEV_1/FVC*	*%Predicted FEF_{25-75}*	*Years smoked*	*Smoking history (pack-years)*
NNS	12/7	61.8 (7.5)	98.8 (15.3)	100.1 (15.9)	98.8 (6.8)	94.1 (33.5)	--	--
NLFS	4/6	54.2 (10.7)	92.6 (8.6)	95.3 (10.7)	97.2 (4.7)	87.5 (15.7)	33.2 (13.2)	33.8 (18.2)
COPD-CS	2/9	64.7 (7.2)	**76.8 (14.2)**	97.1 (17.0)	***79.5 (9.3)**	***42.8 (12.2)**	31.2 (10.4)	***76.8 (14.2)
COPD-ES	2/4	63.7 (8.9)	**75 (10.35)**	92.5 (17.4)	***82.2 (13.1)**	***38.7 (9.5)**	44.2 (11.8)	*†41.7 (20.7)
COPD	4/13	64.4 (7.6)	***76.2 (12.7)**	95.5 (16.8)	***80.4 (10.5)**	***40.2 (10.3)**	35.8 (12.3)	*64.4 (23.7)

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TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

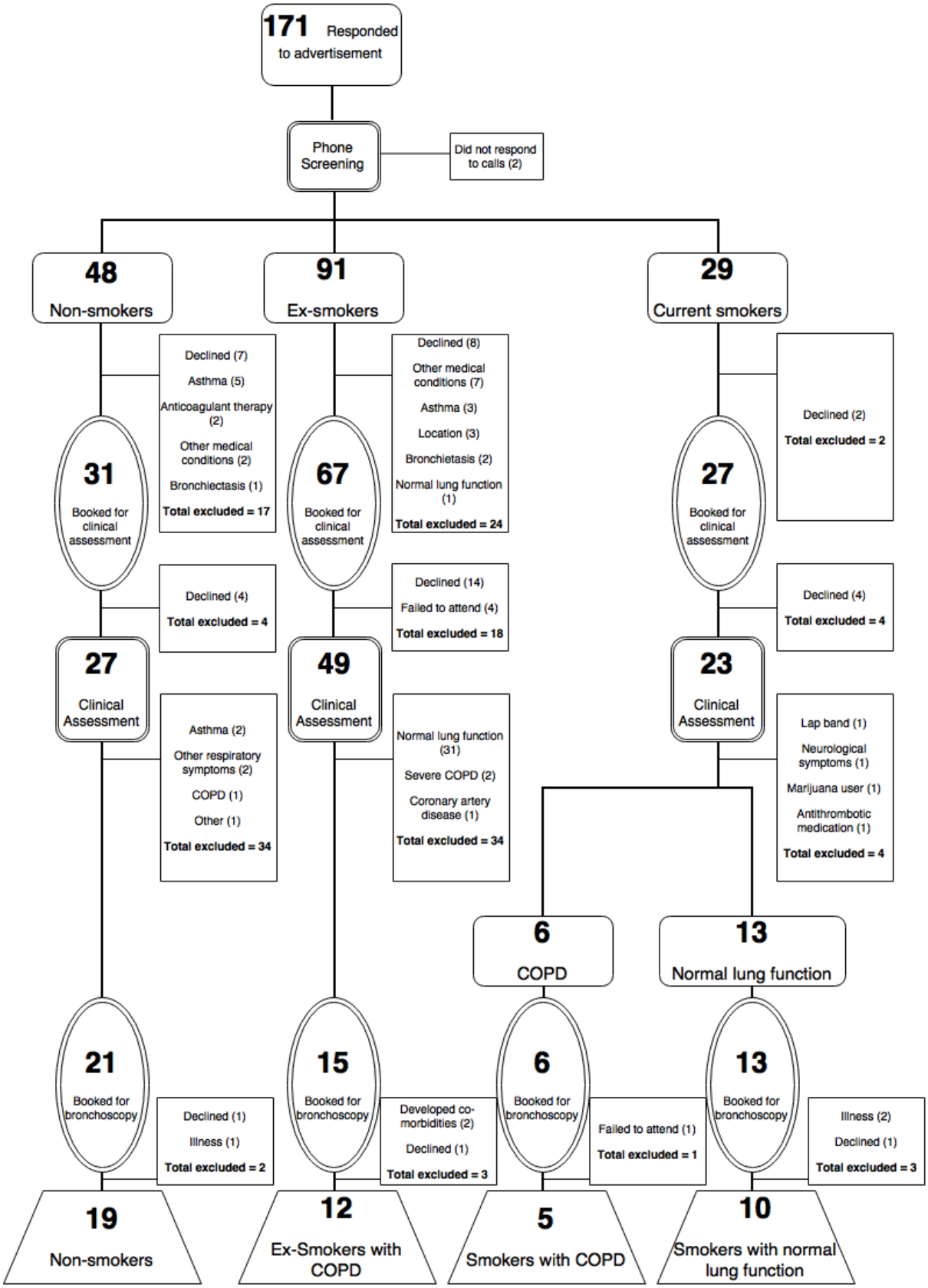

Figure 2.2-1: Flowchart of volunteer recruitment and retention.

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

2.3. General cell culture methods

2.3.1. Flask, slide and plate coating procedure

Flasks were coated with a solution of 1:30 bovine collagen (Type I; Life Technologies, cat# A10644-01) in PBS (Life Technologies, cat# 10010-023). The collagen mixture was applied to the culture surface of the flasks, plates or slides used, and placed in a humidified incubator at 37°C and 5% CO_2 for a minimum of an hour and a maximum of two hours. Following incubation, the collagen solution was removed by tapping the culture vessel hard on its side and aspirating or pouring the coating media off. The culture surface was washed two times with sterile PBS, except in the case of eight-chambered slides, where no washing occurred due to the small surface area and good removal of the collagen.

2.3.2. EMT induction methods

2.3.2.1. Exposure to transforming growth factor-β1 (TGF-β)

The transforming growth factor β-1 (TGF-β) stock solution (Abcam (Sapphire), cat# ab50036; prepared in water) was dissolved in BEGM at a ratio of 1:1,000 to give a working concentration of 10ng/µl, which would be more than sufficient to induce EMT [91]. All cells had their media poured off and disposed of. The TGF-β solution was applied to the cells in the amounts as follows: T-25 flasks required 4ml of solution, T-75 flasks 7ml of solution, 6WPs 2ml of solution, 12WPs 1ml of solution, 24WP 0.5ml solution and ECSs 200µl of solution.

Cells that were not being treated had fresh BEGM applied in the same amounts. All cells were incubated at 37°C and 5% CO_2 in a humidified incubator. Images were taken on a DMIL (Leica) microscope using a Leica DFC320 camera and the imaging program FireCam (version 3.4.1) at 4x and 10x objective magnification both prior to and at the conclusion of treatment. Treatment was commenced when cells were approximately 80-90% confluent for BEAS-2Bs, and five days after being thawed for pHBECs, and was maintained for 72 hours.

2.3.2.2. Exposure to cigarette smoke extract (CSE)

Cigarette smoke extract was generously provided by the Edvinsson group at Lund University, Sweden [209]. The extract was transported dried on filter paper – extraction was achieved by soaking the filter paper overnight in DMSO (1ml). For treatment of cells, based on previous dose response work by Jessica Kregor within the author's group, CSE was applied to cells at 0.1% in complete BEGM for four hours. 0.1% CSE worked out as roughly equivalent to

0.3% of a single cigarette and with 0.8mg of nicotine per cigarette [199, 209], an expected total of approximately 7.2µg2µg nicotine in a single exposure. Standardisation of cigarette smoke is, however, difficult due to the variable composition of cigarettes between brands, as well as the variability in what individuals are exposed to while smoking.

2.3.3. Drug treatments

2.3.3.1. Salmeterol xinafoate

4.5mg of salmeterol xinafoate (Sigma-Aldrich, cat# S5068-10MG) was measured out and diluted in 745µl DMSO (Sigma-Aldrich, cat# D8418-50ML) to obtain a 10mM stock solution. It was stored at room temperature in the dark. Salmeterol has a pharmacological Kd value (the concentration at which the substrate occupies 50% of available receptors) of 1-2nM [210].

2.3.3.2. Fluticasone proprionate

5mg of fluticasone proprionate (Sigma-Aldrich, cat# F9428-5MG) was diluted directly in the bottle with 1,000µl DMSO (Sigma-Aldrich, cat# D8418-50ML) to obtain a 10mM stock solution. It was stored at room temperature in the dark. Fluticasone has a pharmacological Kd value of 42.5-19.5 nM [211].

2.3.3.3. Tiotropium bromide

5.3mg Tiotropium bromide monohydrate provided by Boehringer Ingelheim was diluted in 1,120µl DMSO (Sigma-Aldrich, cat# D8418-50ML) to obtain a 10mM stock dilution. It was stored at room temperature in the dark.Tiotropium has a pharmacological Kd value of 0.1-0.3 nM [212].

2.3.3.4. Treatment procedure

Unless otherwise stated, all drugs were delivered at a 10µM dilution (1:1000 dilution from stock) in cell growth medium for 24 hours. This dosage was decided upon following personal communication with Dr. Mathew Eapen, also in the Centre for Research ExcellenceCRE,

who had previously worked in industry and recommended the dosage as a standard starting point. The dosage was also consistent with the experiences in early-career pharmacological studies on airway tissue *in vitro* of Professor E.H. Walters, and the concentration is far above the Kd of the three drugs, ensuring saturation of receptors. Drugs were started 20 hours prior to treatment with cigarette smoke extract (CSE) or after 48 hours of treatment with TGF-β (if applicable) and were applied concurrent with the treatments. Controls for these samples had DMSO added to the growth medium at 0.3% v/v.

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA – *long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

2.3.1. Collection of supernatant for ELISA

24 hours before the end of treatment the culture media was removed and replaced with fresh media. If treatments were less than 24 hours, no replacement occurred. Following the remainder of the treatment, the supernatant from the cells was collected and centrifuged at 1,500g for 10 minutes at 4°C (Beckman-Coulter Allegra X-12R or Centurion Scientific K3 Series centrifuge). Following centrifugation, the supernatant was removed and stored in 1ml aliquots at -80°C until use.

2.4. Cell culture (PHBECs)

NOTE: Prior to cryopreservation, BEGM contained all supplements supplied in the Lonza SingleQuots kit (cat# CC3170), in addition to 2.5µg/ml Amphotericin B (AmpB) (Sigma-Aldrich, cat# A2942-50ML) and 1 unit penicillin and 1ug/ml streptomycin (using a stock of penicillin/streptomycin (penstrep) (Sigma-Aldrich, cat# P4333-100ML)). Following cryopreservation and thawing (passage 2 and beyond), BEGM contained all supplements supplied in the Lonza SingleQuots kit (cat# CC3170) without additional antibiotics.

2.4.1. General methods

2.4.1.1. Collection of samples

Brushings were used to culture PHBECs. A small wire brush was passed down through the tube attached parallel to the bronchoscope, and was scraped repeatedly against the wall of the bronchi. The brush was withdrawn and the end placed in BEGM. Four brushes were collected, each placed in separate tubes. Samples and culture media were exposed to air for the least amount of time possible. All collection and transport occurred at ambient temperatures.

2.4.1.2. Culture set up

Once collected, the brushes were transferred to the Menzies Research Institute. The brushes were vigorously agitated within the media to place as many cells as possible into suspension, before the brushes themselves were placed in a coated T-25 flask with BEGM (4ml). The media containing the cells was centrifuged in a Heraeus Megafuge 2.0 centrifuge at 1,500rpm for five minutes. The supernatant was discarded and the cell pellet resuspended in BEGM

(1ml). Each cell pellet was placed into a separate coated T-25 flask, with additional BEGM (4ml). All flasks were incubated at 37°C and 5%CO_2 in a humidified incubator. These cells were designated passage number 0 (p0).

2.4.1.3. Maintenance

Cells were fed two to three times weekly. 'Feeding' consisted of aspirating or pouring off the old, used media and applying fresh media. T-25 flasks required 4ml of BEGM, T-75 flasks 7ml of BEGM, 6-well plates (WPs) 2ml of BEGM, 12WPs 1ml of BEGM, 24WP 0.5ml BEGM and ECSs 200µl of BEGM.

Upon cells becoming approximately 80-100% confluent, images were taken on a DMIL (Leica) microscope using a Leica DFC320 camera and the imaging program FireCam (version 3.4.1) at 4x and 10x objective magnification. The media was discarded or collected for ELISA (see 2.10.1), and trypsin/EDTA (Sigma-Aldrich, 0.25% trypsin, 0.02% EDTA, cat# T4049) was applied to the cells. The cells were incubated at 37°C and 5% CO_2 in a humidified incubator until they were detached from the culture surface, at which time BEGM equal to four times the volume of trypsin/EDTA (Sigma-Aldrich, 0.25% trypsin, 0.02% EDTA, cat# T4049) was added to the cells. The cell solution was swirled in the flask to encourage all the cells to detach, before being centrifuged at 1,500rpm for five minutes. The supernatant was discarded and the cell pellet resuspended in BEGM. For cells at p0, resuspension was in BEGM (1ml); for other passage numbers the volume varied depending on the intended use (see the appropriate section for further details). The cells were seeded into the appropriate coated culture vessels and the passage number increased by one. Cells at p0 in a single T-25 flask were seeded into two T-75 flasks and became p1 cells, for example.

2.4.1.4. Cryopreservation

Cells were trypsinised as described in 2.4.1.3. Following centrifugation the cell pellet was resuspended in cryoprotectant comprised of 10% DMSO in FBS. Cell pellets obtained from T-25 flasks were resuspended in 2ml of cryoprotectant, and cell pellets from T-75 flasks in 3ml. The only exception was when half of the flask was taken for RNA extraction, at which point cells were resuspended in 1ml and 2ml respectively. The cell solution (1ml) was placed into Nalgene 1.5ml cryovials (Sigma-Aldrich, cat# V4757) and placed in a Nalgene Mr. Frosty cryo-cooler device (Sigm-Aldrich, ca# C1562). The Mr. Frosty was stored at -80°C overnight (occasionally for longer, no more than 72 hours, if noted), after which the cryovials were transferred to vapour phase liquid nitrogen storage.

2.4.1.5. Reviving cryopreserved cells

Frozen cells were retrieved from the liquid nitrogen storage on ice, and then rapidly thawed by hand, or in a water bath heated to 37°C for large numbers of vials. The cells were quickly placed into excess BEGM (9ml) and centrifuged at 1,500rpm (Heraeus, Megafuge 2.0) or 130g (Beckman Coulter, Allegra X-12R) for five minutes. The supernatant was discarded, and the cells resuspended in BEGM (1ml). Cells were either seeded into one coated T-75 flask with BEGM (7ml) or into three T-25 flasks with BEGM (4ml). Flasks were incubated at 37°C and 5% CO_2 in a humidified incubator.

2.4.2. Culture for immunocytochemistry

2.4.2.1. Cell culture on coverslips

Coverslips were placed in a 12-well plate, one slip per well. Bovine Type I collagen (Life Technologies, cat# A10644-01) was dissolved to a final dilution of 1:30 in ethanol (30% in water). The collagen solution was applied to the bottom of the wells in an amount sufficient to cover the slips and the surface of the well and left overnight to evaporate. However, due to issues with cell growth on the coverslips and overall difficulty with staining and handling of the coverslips, this method was abandoned.

Cells were brought up from storage (2.4.1.5), grown and passaged as described in 2.4.1.3. Following trypsinisation, cells were seeded into the coated wells containing coverslips (2.3.1) and incubated with fresh BEGM at 37°C and 5% CO_2. Cells were grown until 95-100% confluency before being used for immunocytochemistry.

2.4.2.2. Cell culture on eight-chambered slides

Cells were brought up from storage (2.4.1.5) and seeded into the wells coated with 1:30 bovine collagen (Type I; Life Technologies, cat# A10644-01) in PBS (Life Technologies, cat# 10010-023) (2.3.1). A single cryovial (1ml of cells) was used to seed 9 wells. Cells were incubated with fresh BEGM at 37°C and 5% CO_2 for five days, and either treated as described in section 2.3.2 and 2.3.3 or left untreated. Following treatment, cells were prepared as described in section 2.9.

2.5. Cell culture (PBFs)

NOTE: DMEM was supplemented with 10% FBS and contained 2.5µg/ml AmpB (Sigma-Aldrich, cat# A2942-50ML) and 1 unit penicillin and 1ug/ml streptomycin (Sigma-Aldrich, cat# P4333-100ML).

2.5.1. Collection of samples

A bronchoscopy was performed as described in 2.4.1.1, with a single biopsy taken and placed in DMEM supplemented with 10% FBS.

2.5.2. Culture set up

Biopsies were 'wounded' by gently cutting with a scalpel blade, passing through a 1000µl pipette tip or abrading on a filter. Wounded biopsies were then placed in a coated T-25 flask with DMEM supplemented with 10% FBS (4ml) and incubated at 37°C and 5% CO_2 in a humidified incubator.

2.5.3. Maintenance

Upon formation of a monolayer extending some distance from the biopsy, the media was decanted and trypsin/EDTA applied (Sigma-Aldrich, 0.25% trypsin, 0.02% EDTA, cat# T4049). The cells were incubated at 37°C and 5% CO_2 in a humidified incubator for fifteen minutes and DMEM with 10% FBS was added (4ml). The cells were centrifuged at 1,500rpm in a Heraeus, Megafuge 2.0 centrifuge for 10 minutes, following which the cell pellets were resuspended in DMEM with 10% FBS (1ml) and placed in a fresh coated T-25 flask with DMEM/FBS (4ml). Cells were incubated at 37°C and 5% CO_2 in a humidified incubator, however no further growth was observed and the cells were abandoned.

2.5.4. Brief discussion of fibroblasts

Attempts were made to culture fibroblasts from biopsies collected with the brushings and stored in DMEM, however all but one sample failed to proliferate fibroblasts, and following passage that sample failed to grow and died. A total of two flasks taken from brushings became spontaneously overrun with what appeared at the gross phenotypic level to be fibroblasts (Figure 3.2-1), one flask at passage zero and, interestingly, one flask at passage 2 following cryopreservation and thawing, both growing in bronchial epithelial growth media (BEGM). The flask at passage 0 was given to Mathew Eapen, a fellow PhD student who was intending to culture fibroblasts as part of his study, however they subsequently died. The flask which was overrun at passage 2, which was especially interesting since it was taken from a different volunteer from the initially overrun flask and both prior to freezing and for some time after thawing appeared phenotypically identical to the other epithelial cells, was grown successfully in BEGM and frozen in the same manner as the epithelial cells (2.4.1.4). No tests were performed on these cells, although they remain in storage.

It was unclear why culturing fibroblasts from explants was unsuccessful – potentially, supplementation of the media with additional serum or growth factors may be recommended. In cases where the fibroblasts overtook the epithelial cell cultures, they proved to be hardy and prolific, however in these cases it is probable that the cultures would still remain mixed. If required to utilise these fibroblasts, it would be recommended that some form of cell sorting, possibly using flow cytometry, be performed to ensure pure cultures of fibroblasts without epithelial cell contamination. In conclusion, the attempted culture of paired

fibroblasts from biopsies matched to epithelial cultures was a failure, although the reasons for the failure remain unknown.

Useful abbreviations

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COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA – *long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

2.6. Cell culture (BEAS-2B)

NOTE: BEGM contained all supplements supplied in the Lonza SingleQuots kit (cat# CC3170) with no additional antibiotics.

2.6.1. Creating a working stock of BEAS-2B cells

2.6.1.1. Coating procedure

The flasks for this procedure were coated with FCS mix, which was a 0.01mg/ml fibronectin, 0.03mg/ml collagen-1, 0.01mg/ml bovine serum albumin mix made up in culture growth media (bronchial epithelial growth media without the AmpB SingleQuot). The mix (4ml) was placed on the flasks for 2 hours at 37°C, 5% CO_2 then removed and the flasks were air-dried overnight in the biosafety hood before use.

2.6.1.2. Thawing and culture maintenance

A single vial of BEAS-2B cells at passage 4 (p4) was obtained from the American Type Culture Collection (ATCC, cat# CRL-9609) and stored in vapour phase liquid nitrogen. The cells (1ml) were rapidly thawed, placed in bronchial epithelial growth media (9ml, BEGM, without the AmpB SingleQuot) centrifuged at 1,500rpm for 10 mins (Beckman-Coulter, Allegra X-12R), following which the cells were resuspended in fresh BEGM and placed into a single T-75 flask (Corning via Sigma-Aldrich, cat# CLS430641) coated with FCS in BEGM (7ml), as per the commercial instructions for the cells. The thawing procedure was the same for subsequent passages of cells.

During growth, the cells were incubated at 37°C, 5%CO_2 until they reached approximately 90% confluence, with BEGM (7ml) being replaced every Monday and Friday. Upon reaching

90% confluence, the media was removed from the cells and a trypsin/EDTA/polyvinyl pyrroline (2ml, Sigma-Aldrich, 0.25% trypsin, 0.02% EDTA, cat# T4049; Sigma-Aldrich, 0.5% PVP, cat# P0930) solution was applied to the cells until they detached from the flask, as per commercial instructions. The cell solution was placed in BEGM (8ml) and centrifuged at 130g for 5 mins (Beckman-Coulter, Allegra X-12R), before being resuspended in BEGM (7ml) and split into seven coated T-75 flasks. Cells were grown to passage 5 and all flasks were frozen down to create a master stock of cells, with each vial containing cells at the concentration of $1x10^5$cells/ml.

From the master stock, a single vial was thawed. The vial was thawed and grown as described above and passaged into seven T-75 flasks. Those flasks were then grown, passaged and the cells cryopreserved, at a final concentration of $1x10^6$cells/ml.

2.6.1.3. Cryopreservation

Following trypsinisation, the cells were centrifuged at 130g for 5 mins (Beckman-Coulter, Allegra X-12R), before being resuspended in a cryoprotectant mix comprised of 10% DMSO and 90% BEGM. The cells were resuspended to a concentration of $1x10^5$cells/ml for the master stock (due to a calculation error) and $1x10^6$cells/ml for the working stock. Vials were then placed in a -80°C freezer in a Nalgene Mr. Frosty cryo-cooler (Sigma-Aldrich, cat# C1562) overnight before being transferred to vapour phase liquid nitrogen for storage.

2.6.2. General methods

2.6.2.1. Reviving cryopreserved cells

Frozen cells were retrieved from the liquid nitrogen storage on ice, and then rapidly thawed by hand. The cells were quickly placed into excess BEGM (9ml) and centrifuged at 300g for five minutes. The supernatant was discarded, and the cells resuspended in BEGM (1ml). Cells were seeded into one coated T-75 flask with BEGM (7ml) and incubated at 37°C and 5% CO_2 in a humidified incubator.

2.6.2.2. Maintenance

Cells were passaged twice a week upon reaching approximately 80% confluence. The old media was removed from the cells and trypsin/EDTA/PVP applied (0.25% trypsin, 0.02% EDTA, 0.5% PVP). During 2016/17, cell growth was greatly diminished and cells were passaged approximately once a month, upon reaching 80-90% confluence.

Cells were incubated at 37°C and 5% CO_2 in a humidified incubator until most cells had detached from the culture surface. BEGM equal to four times the amount of trypsin/EDTA/PVP was added, and the solution swirled gently to encourage any remaining cells to detach. The cell suspension was centrifuged at 300g in an Allegra 12-XR (Beckman Coulter) centrifuge for five minutes. Cells were split one in seven to seed a new coated T-75 flask, with the remainder used to seed coated culture vessels appropriate for the experiments to be performed (see the relevant sections for more detail).

During experimentation, cells were not synchronised in cell cycle by serum starvation, however cells were utilised following a set period of growth after passaging (five days) and every attempt was made to ensure that the cells were at similar confluence, as judged by eye.

2.6.2.3. Cryopreservation

Cells were trypsinised and centrifuged as described in 2.6.2.2. Following centrifugation, the cells were resuspended in cryoprotectant comprised of 7.5% DMSO and 92.5% BEGM to an approximate concentration of $1x10^6$ cells/ml. The cell solution (1ml) was placed into Nalgene 1.5ml cryovials (Sigma-Aldrich, cat# V4757) and placed in a Nalgene Mr. Frosty cryo-cooler device (Sigma-Aldrich, cat# C1562). The Mr. Frosty was stored at -80°C overnight, after which the cryovials were transferred to vapour phase liquid nitrogen storage.

2.6.3. Culture for RNA and intra-cellular protein extraction

Cells were brought up from storage (2.6.2.1), grown and passaged as described in 2.6.2.2. Following trypsinisation, cells used for RNA extraction were resuspended in ice cold PBS (1ml; Life Technologies, cat# 10010-023). Cells were centrifuged at 130g for five minutes and RNA and protein was extracted following the procedure described in 2.8.1.

2.6.4. Culture for immunocytochemistry

Cells were brought up from storage (2.6.2.1) and seeded a single T-75 flask coated with 1:30 bovine collagen (Type I; Life Technologies, cat# A10644-01) in PBS (Life Technologies, cat# 10010-023) (2.3.1). Cells were incubated with fresh BEGM at 37°C and 5% CO_2 until they reached 80% confluence and then were passaged as described in section 2.6.2.2. Cells were split with 2/7 of the total seeded into 2x T-75 flasks coated with 1:30 bovine collagen (Type I; Life Technologies, cat# A10644-01) in PBS (Life Technologies, cat# 10010-023) (2.3.1) and the remainder seeded into 6x ECS. Cells were incubated with fresh BEGM at 37°C and 5% CO_2. The cells in ECSs were grown for five days and either treated as described in sections 2.3.2 and 2.3.3 or left untreated. Following treatment, cells were prepared as described in section 2.9.

Further cells for immunocytochemistry were obtained from the continued T-75 flasks, which were maintained as described in section 2.6.2.2.

2.7. Biopsies

2.7.1. Collection, processing and storage

Biopsies were collected as described in section 2.2.1 and placed in 4% paraformaldehyde at room temperature. The biopsies were fixed at room temperature for 2 hours before being removed from the paraformaldehyde, sandwiched between folded filter paper and then between two pieces of foam, inserted into a plastic embedding cassette and placed in 50% ethanol. The biopsies were stored in a sealed container in 50% ethanol overnight, before being transferred into a Leica Autoprocessor ASP200S, where they were processed using the following procedure:

Table 2.7-1: Protocol settings for the Leica ASP200S autoprocessor for fixing bronchial biopsies. *All steps were set with the fast drain time (80 seconds). P/V cycles are filling (vacuum) and draining (pressure).*

Reagent	*Temperature (°C)*	*Time (minutes)*	*Pressure/Vacuum (kPa)*
70% ethanol	37	20	--
95% ethanol	37	20	--
100% ethanol	37	20	--
100% ethanol	37	10	--
100% ethanol	37	10	--
100% ethanol	37	20	--
Xylene	37	40	--
Xylene	37	40	35/-70
Paraplast wax	60	15	35/-70
Paraplast wax	60	25	35/-70
Paraplast wax	60	40	35/-70

Following processing the biopsies were embedded in Surgipath Paraplast wax (Leica) using an embedding station to melt, dispense and chill the wax. Generally three to four biopsies were embedded out of the four collected at bronchoscopy, however more or less were sometimes obtained due to breakdown and fragmentation of the tissue during collection and processing.

Prior to cutting, biopsies were stored at 4°C. Cut sections were stored at room temperature.

2.7.2. Detection of intra-cellular protein

Biopsies were cut to a thickness of 3μm by Steve Weston and placed on slides. Sections were dewaxed by placing slides in xylene (100%) for 5 minutes, following which slides were transferred to fresh xylene (100%) for 5 minutes. Slides were then placed in ethanol solutions of 100%, 95% and 70% ethanol for five minutes each time, in the specified order, before being transferred to tap water. Sections then underwent antigen retrieval using High pH FLEX Target Retrieval Solution (pH9; Dako, cat# K8004) in a Dako PT-Link at 97°C for 15 minutes.

Following heat retrieval, slides were briefly rinsed with deionised water and placed in the Dako Autostainer Plus, running the following program:

Table 2.7-2: Dako autostainer program for immunohistochemistry.

Step name	*Reagent (600µl)*	*Time (min)*
Rinse	Tris-HCl	--
Permeablisation	3% H_2O_2	20
Rinse	Tris-HCl	--
Rinse	Tris-HCl	--
Primary antibody	See Table 2.12-1	90
Rinse	Tris-HCl	--
Rinse	Tris-HCl	--
Mouse HRP	Envision Mouse HRP	30
Rinse	Tris-HCl	--
Rinse	Tris-HCl	--
DAB+	DAB+	10
Rinse	Tris-HCl	--
Rinse	Distilled water	--

For negative control slides, the primary antibody was replaced with an isotype control consisting of species appropriate IgG. Slides were rinsed with water and placed in Meyer's haematoxylin for 5 minutes. Excess haematoxylin was rinsed off under running tap water until the water ran clear rather than blue or purple. Haematoxylin staining was fixed by placing the slides into ammoniated water for a minimum of 3 minutes.

Following nuclear staining, slides were dehydrated by moving them through a series of dehydrating solutions. In order, the solutions were: 95% ethanol, 100% ethanol (two different batches), 100% xylene (two different batches). The slides were exposed to the initial three solutions (ethanol-containing) for five minutes each and were exposed to the final two solutions (xylene) for two minutes per solution. The slides were manually mounted using Dako hard-set mounting media (Dako, cat# CS703), with unmounted slides left in the final xylene bath until mounting. Mounted slides were air-dried overnight at room temperature.

2.7.3. RNAScope

RNAScope was attempted on biopsies collected in section Collection, processing and storage2.7.1 using the RNAScope 2.5HD Detection Reagent BROWN kit (Advanced Cell Diagnostics, cat# 322370). The procedure was performed according to the manufacturer's instructions, and utilised Peptidylprolyl Isomerase B (PPIB) as the positive control probe (Advanced Cell Diagnostics, cat# PN 313901). Briefly, the samples were baked for 1 hour at 60°C before being deparaffinised by placing the slides sequentially into two xylene (100%) and then two ethanol (100%) baths, with the xylene baths lasting for 5 minutes and the ethanol baths for 1 minute.

The slides were air dried, and 8 drops of RNAScope hydrogen peroxide were added to the sample and were incubated at room temperature for 15 minutes. Following incubation the hydrogen peroxide was removed and the slides washed twice in distilled water. The slides were then submerged in boiling RNAScope Target Retrieval Solution. In the initial attempt, slides were submerged for 15 minutes, however the Target Retrieval Solution only returned to the boil for the final five minutes of submersion. Two additional attempts were made to optimise the RNAScope, one submerging the slides for 30 minutes and the other submerging the slides and waiting until the Target Retrieval Solution has returned to boil before starting the 15 minute timer.

Following target retrieval the slides were washed in distilled water four times before being rinsed with ethanol (100%) and allowed to air dry. A hydrophobic barrier was drawn around the sample using an Immedge hydrophobic barrier pen and allowed to dry overnight.

Slides were placed in the HybEZ oven with 5 drops of RNAScope Protease Plus and incubated at 40°C for 30 minutes. The Protease Plus was removed from the slide following incubation and the slides were washed four times in distilled water.

Slides were hybridised with the probes as follows in the HybEZ oven at 40°C:

Table 2.7-3: RNAScope hybridisation and amplification incubation periods. *All incubations except for the Amp6 step were performed in the HybEZ oven.*

Probe	*Exposure time (min)*	*Temperature (°C)*
PPIB/Negative control	120	40
Amp1	30	40
Amp2	15	40
Amp3	30	40
Amp4	15	40
Amp5	30	40
Amp6	15	Room temperature

Between each step in Table 2.7-3 slides were washed twice for 2 minutes in RNAScope Wash Buffer before continuing with the next step. Following the washes after Amp6 treatment, the RNAScope DAB-A and DAB-B reagents were combined, two drops of each reagent per sample on the slide, and then applied to the slides. Slides were incubated for 10 minutes at room temperature before being rinsed four times with tap water. Following the rinses, the slides were placed in Meyer's haematoxylin for 5 minutes. Excess haematoxylin was rinsed off under running tap water until the water ran clear rather than blue or purple. Haematoxylin staining was fixed by placing the slides into ammoniated water for a minimum of 7 minutes.

Following nuclear staining, slides were dehydrated by moving them through a series of dehydrating solutions. In order, the solutions were: 95% ethanol, 100% ethanol (two different batches), and 100% xylene. The slides were exposed to each solution for five minutes. The slides were manually mounted using Dako hard-set mounting media (Dako, cat# CS703), with unmounted slides left in the final xylene bath until mounting. Mounted slides were air-dried overnight at room temperature.

However, despite attempts at optimisation described above RNAScope failed to produce DAB staining of the housekeeping gene in sample biopsies, although staining was present in the HeLa cell pellet (Advanced Cell Diagnostics, PN 310045) provided as a control, indicating that there was no issue with the procedure itself.

2.7.4. Analysis of staining

2.7.4.1. ImageJ vs. Image Pro Plus

Previous work within the group had been done using Image Pro Plus software to analyse biopsy images, however the free-to-use ImageJ software had a plugin designed for isolating DAB staining in colour images, allowing for easy quantification via the in-built 'threshold' function. A brief assessment of the two programs was undertaken to determine whether one was better suited to the task of analysis.

An example of high-staining (cytokeratin) and low staining (S100A4) was used. As seen in Figure 2.7-1 and Figure 2.7-2, the percentage of the epithelium stained was approximately the same between both programs, with ImageJ being slightly more sensitive to low, discrete staining such that seen as in S100A4 stained biopsies. As both programs appeared similar in capability, the decision was made to utilise ImageJ and its successor FIJI Is Just ImageJ (FIJI) for analysis of immunohistochemical and immunocytochemical staining. Analysis of immunohistochemical staining was undertaken using ImageJ and the IHCToolbox plugin [210].

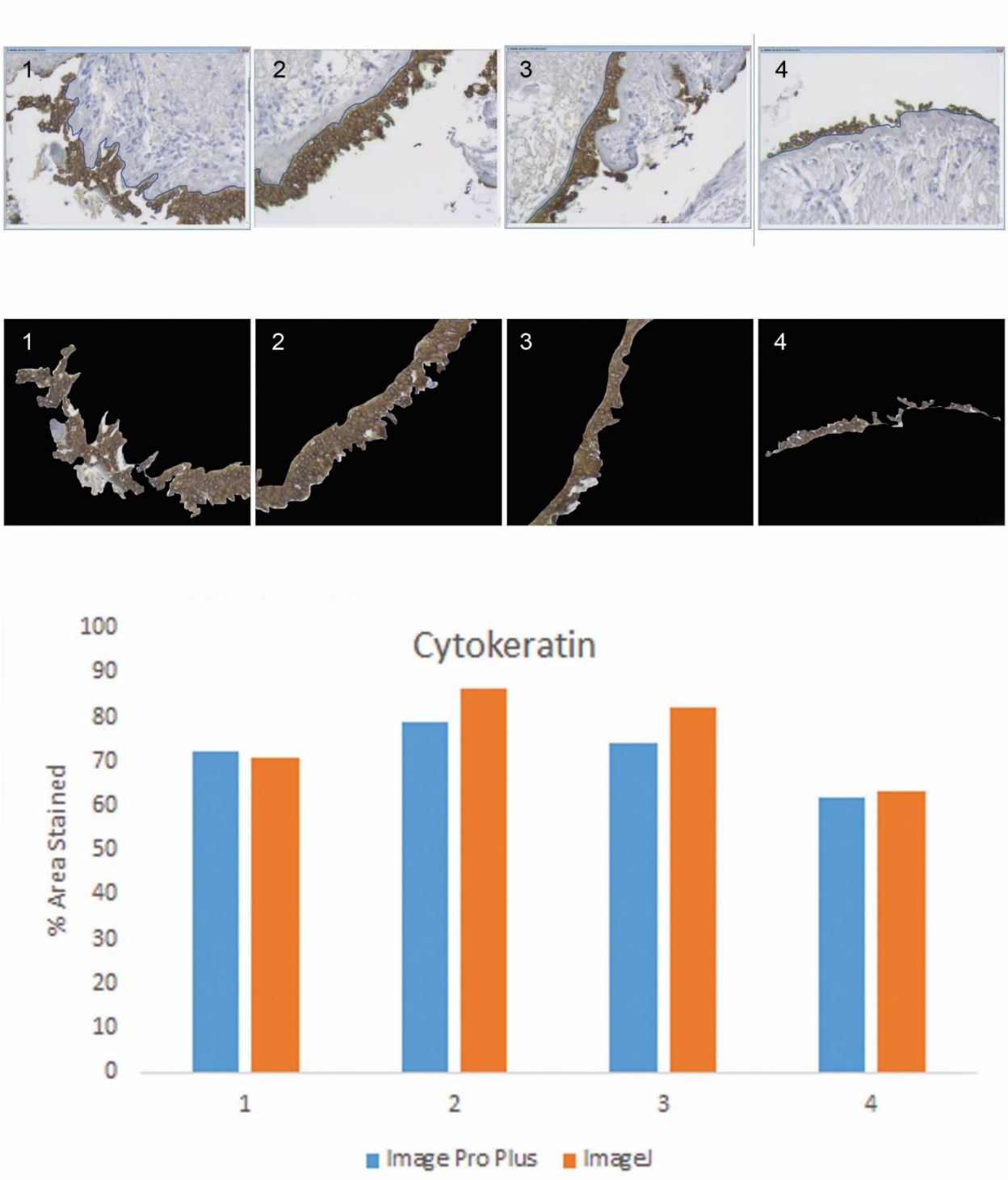

Figure 2.7-1: A comparison of cytokeratin staining in airway epithelium as measured using Image Pro Plus software versus the ImageJ IHCToolbox. *Four random images were selected from a single sample and the amount of staining quantified in both Image Pro Plus and ImageJ. Images were first measured in Image Pro Plus, where the epithelial area of interest was defined – every effort was taken to match the area in ImageJ. The blue line in the top row of images denotes the length of the reticular basement membrane, as seen in Image Pro Plus. The bottom row of images shows the total area of epithelium measured, taken from ImageJ. Area stained is expressed as a percentage of total area of the epithelium.*

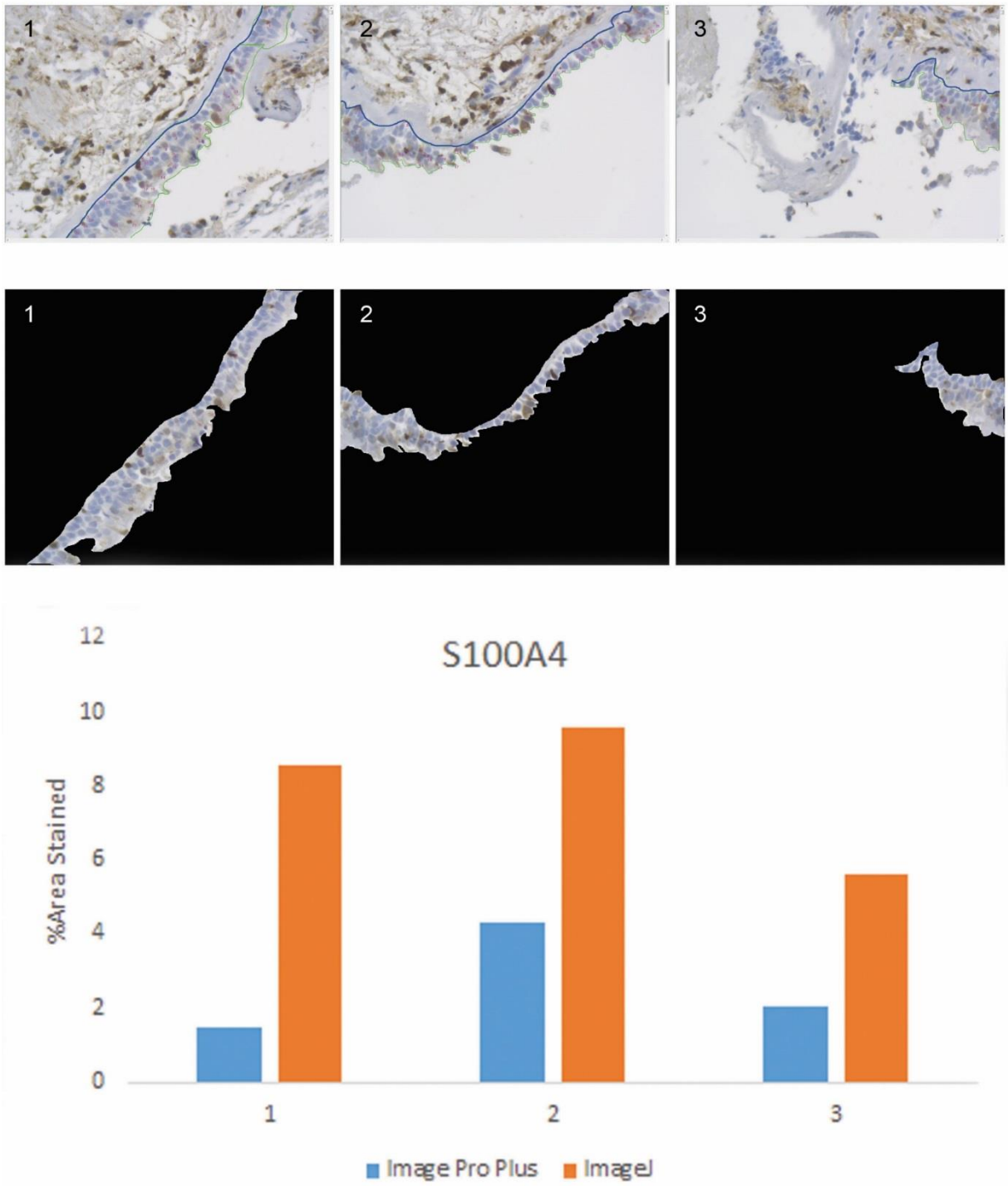

Figure 2.7-2: A comparison of S100A4 staining in airway epithelium as measured using Image Pro Plus software versus the ImageJ IHCToolbox. *Three random images were selected from a single sample and the amount of staining quantified in both Image Pro Plus and ImageJ. Images were first measured in Image Pro Plus, where the epithelial area of interest was defined – every effort was taken to match the area in ImageJ. The blue line in the top row of images denotes the length of the reticular basement membrane, as seen in Image Pro Plus. The bottom row of images shows the total area of epithelium measured, taken from ImageJ. Area stained is expressed as a percentage of total area of the epithelium.*

2.8. q-PCR analysis

2.8.1. RNA extraction

2.8.1.1. Promega ReliaPrep

Following trypsinisation, cells used for RNA extraction were resuspended in ice cold PBS (1ml; Life Technologies, cat# 10010-023). RNA was extracted according to the manufacturer's instructions. Brieflyie, cells were centrifuged at 1,300g for five minutes (Heraeus Pico21 Centrifuge). Combined thioglycerol (2.5µl) and buffer BL (247.5µl) was added to the resulting cell pellet. Isopropanol (85µl) was added to sample, mixed well, and the resulting mixture was transferred to a ReliaPrep mini-column.

The sample was centrifuged at 13,000g for 1 minute and RNA wash buffer (500µl) was added to the column membrane. The sample was centrifuged for a further minute at 13,000g while Yellow Core Buffer (24µl), $MnCl_2$ (3µl) and DNaseI (3µl) were combined. The DNase solution was added to the column membrane and incubated at room temperature for 15 minutes.

Following incubation, column wash buffer (200µl) was added to the column and the sample was centrifuged at 13,000g for 1 minute. RNA wash buffer (300µl) was added to the column and centrifuged through at 13,000g for 1 minute. Additional RNA wash buffer (500µl) was added to the column and the sample centrifuged at 21,100g for 2 minutes to dry the column membrane. Nuclease free water (30µl) was applied to the column and the RNA was eluted into a clean tube and stored at -80°C until use.

RNA was quantified using a Nanodrop8000 and diluted to a concentration of 50ng/µl prior to use, or was used undiluted if the initial concentration was less than 50ng/µl.

Samples used for pilot data and testing were collected utilising this method, however in order to collect both protein and RNA from primary cell samples with limited availability, for the main study RNA was collected using the QIAgen RNA/Protein AllPrep kit, as described below.

2.8.1.2. QIAgen RNA/Protein AllPrep

Cells were grown in 12-well plates as described in section 2.4.1 for pHBECs and 0 for BEAS-2B cells. RNA extraction was performed using the AllPrep RNA/Protein kit (QIAgen, cat# 80404), as according to the manufacturer's instructions. Briefly, the cells were washed with PBS and buffer APL (200µl) was added to each well and allowed to incubate at room temperature for five minutes. Following incubation the well bottom was scratched vigorously with the tip of a micropipette and the lysate transferred to an AllPrep spin column after being pipetted up and down several times in order to further help lysis. The homogenate was spun through the column at 8,500g (Heraeus Pico21 centrifuge). For the first 24 samples, protein extraction was performed first, however for all subsequent samples RNA extraction was performed first.

Buffer RLT (350µl) was added to the AllPrep spin column and centrifuged at 8,000g for 1 minute (Heraeus Pico21 centrifuge). Ethanol (70%, 350µl) was added to the resultant flow through and mixed, before the entire volume was transferred to an RNeasy spin column and centrifuged at 8,000g for 1 minute. Buffer RW1 (700µl) was added to the column and centrifuged at 8,000g for 30 seconds. Buffer RPE (500µl) was added to the column and centrifuged at 8,000g for 30 seconds, two times in succession. The empty spin column was then centrifuged at 21,100g for 2 minutes to dry the column, before nuclease free water (30µl) was added to the column membrane. RNA was eluted by centrifuging at 8,000g for 1 minute and was stored at -80°C until use.

RNA was quantified using a Nanodrop8000 and diluted to a concentration of 50ng/µl prior to use, or was used undiluted if the initial concentration was less than 50ng/µl.

2.8.2. Reverse transcription

Reverse transcription of RNA samples was performed according to the manufacturer's instructions for the GoScript Reverse Transcription kit (Promega, cat# A5001). Briefly, a reverse transcription master mix containing (per sample) nuclease free water (5µl), 5x Reaction Buffer (4µl), $MgCl_2$ (5µl) and 1µl each of nucleotide mix, random primers, primer oligo(dT)s and reverse transcriptase was mixed. The master mix (18µl) was added to the RNA (2µl, 100ng) and the mixture heated on a BRESATEC PRC-100 heat block. The heating steps were:

- 25°C for 5 minutes.
- 42°C for 60 minutes.
- 70°C for 15 minutes.

Samples were then diluted 1:100 to achieve a final concentration of cDNA of 1ng/µl and stored at -20°C until use.

2.8.3. q-PCR

Quantitative real-time PCR was performed on a LightCycler480 (Roche) in 96-well plates. Reactions were 25µl total, consisting of nuclease free water (9µl), QuantiTect SYBR Green PCR Master Mix (12.5µl; QIAgen, cat# 204145), QuantiTect Primer Assay (2.5µl; QIAgen, see TABLE) and cDNA (1µl, 1ng/µl). QuantiTect Primer assays are designed by QIAgen to cross intron-exon boundaries, which removes the need for a no-reverse-transcription control as they cannot amplify gDNA. Although including a control would be best practice, due to limitations on time and samples it was not possible.

Roche Lighcycler settings were as follows:

Table 2.8-1: Roche LightCycler480 qPCR program specifications.

Name of step	*Temperature (°C)*	*Duration*	*Number of cycles*
Activation	95	15 min	1
PCR	94	15 sec	45
	55	30 sec	
	72	30 sec	
Melt Curve	95	--	1

2.8.4. Analysis

Analyses of qPCR data were performed using the method described by Pfaffl [214]. Efficiency of the primer sets was determined from the slope of the line fitted to a standard curve created via serial dilution of a known concentration of sample. Reference samples from the standard curve were run on each plate and the raw data were corrected according to changes in the reference sample compared to the standard curve. All data were normalised to the expression of the housekeeping gene β-actin. In all data sets, each point represents an individual experimental sample. Analysis of the data consisted of a two-way ANOVA applied to the three pHBEC groups treated with drugs, and all other data were analysed with one-way ANOVAs.

2.8.5. Brief discussion of brachyury qPCR

As previously discussed (1.1.2.6), a person having COPD has, irrespective of their current smoking status, a six-fold increased risk of lung cancer compared to a person without COPD [67, 68]. In the large airways, people with late-stage COPD see predominantly squamous cell carcinomas, while people with Stage I COPD tend to see a predominance of adenocarcinomas [16, 72].

Brachyury is a T-box transcription factor which has been shown to be expressed in 41% of primary lung tumours [212]. In the study by Roselli and colleagues, brachyury was expressed by 48% of adenocarcinomas and 25% of squamous cell carcinomas. Brachyury is also expressed in breast and prostate cancers [213-215]. A feature which makes brachyury especially useful to examine potential links between lung cancer and COPD is the fact that it is not expressed at detectable levels in normal lung tissue, or even in the majority of normal tissues outside of the prostate [216], which makes it a neat binary marker. Of further importance to COPD, brachyury is closely associated with an EMT phenotype in breast cancer [213, 217], and may potentially be a link between EMT in chronic airflow limitation which is not yet clinical COPD and the increased risk of lung cancer in COPD.

The initial standard curve for the qPCR failed to show expression of brachyury, except in one sample in the middle of the concentration curve. The source of this peak is unknown but expected to be a random artefact, possibly due to point contamination of the well rather than a true signal based on the fact that higher concentrations of RNA did not show expression. It is highly likely this lack of signal was due to the fact the XPressRef Universal RNA from

QIAgen is sourced from normal, non-diseased cells, and these are unlikely to express brachyury at a detectable level [216].

Several cell lines, including the prostate cancer cell line LNCAP and the breast cancer cell line MDA-MB-231 are known to be susceptible to brachyury-mediated immune responses in their baseline state [214]. A fellow PhD student, Emma Wilkinson, had access to RNA taken from MDA-MB-231 cells, of which she donated a small amount for use as a standard curve. However, despite expected expression of brachyury in this cell line, no expression was seen in qPCR.

A small number of samples, including two at random from the standard curves utilising universal RNA and RNA from the MDA-MB-231 cell line, showed Ct values, however with Ct values of greater than 35 cycles it is unlikely that these signals represented true expression. This was further backed up by the melt curves for the unknown samples, which did not exhibit neat peaks at identical positions on the graph

Primers for brachyury, like all the primer assays used in this study, were obtained commercially from QIAgen and are therefore confirmed to amplify product when present. Optimisation of reaction temperature was not possible for this primer set due to lack of reagents and time, which may have been a contributing factor to the lack of results. Improper annealing temperatures can affect primer binding and reduce or eliminate signal, even when product is present in the sample. It is likely, however, that brachyury was simply not expressed in the samples. As previously discussed, the universal RNA came from non-diseased normal tissue, and thus was unlikely to express brachyury [216], and the paper which was used to suggest the MDA-MB-231 cell line looked at an indirect measure of

protein expression [214], which may not indicate expression at the transcript level. Finally, although COPD is associated with an increased risk of lung cancer, and specifically adenocarcinoma in the large airways [16, 72], where the samples were taken from, brachyury is only expressed in 50% of adenocarcinomas [212] and thus may not be present in the samples. There is also the possibility that brachyury may be expressed only in established adenocarcinomas, and may not be present in cases of non-COPD airflow limitation, hence the lack of signal from the samples.

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

2.9. Immunocytochemistry

2.9.1. Immunocytochemical staining

2.9.1.1. Staining procedure

Media was removed from the cells and replaced with ice-cold acetone (dried with Molecular Sieve type 4A beads (ProSciTech, cat# C830)) for 5 minutes to fix cells. Following fixation, cells were washed with tris-HCl (0.5M, made up as described in section 2.12.1). Heat retrieval of the antigens was performed in High pH FLEX Target Retrieval Solution (pH9; Dako, cat# K8004) in a Dako PT-Link at 97°C for 15 minutes.

Following heat retrieval, slides were briefly rinsed with deionised water and placed in the Dako Autostainer Plus, running the following program:

Table 2.9-1: Dako autostainer program for immunocytochemistry.

Step name	*Reagent (600µl)*	*Time (min)*
Rinse	Tris-HCl	--
Permeablisation	3% H_2O_2	20
Rinse	Tris-HCl	--
Rinse	Tris-HCl	--
Primary antibody	See Table 2.12-2	90
Rinse	Tris-HCl	--
Rinse	Tris-HCl	--
Mouse HRP	Envision Mouse HRP	30
Rinse	Tris-HCl	--
Rinse	Tris-HCl	--
DAB+	DAB+	10
Rinse	Tris-HCl	--
Rinse	Distilled water	--

For negative control slides, the primary antibody was replaced with an isotype control consisting of species appropriate IgG. Slides were rinsed with water and placed in Meyer's haematoxylin for 5 minutes. Excess haematoxylin was rinsed off under running tap water until the water ran clear rather than blue or purple. Haematoxylin staining was fixed by placing the slides into ammoniated water for a minimum of 7 minutes.

Following nuclear staining, slides were dehydrated by moving them through a series of dehydrating solutions. In order, the solutions were: 95% ethanol, 100% ethanol (two different batches), 100% xylene (two different batches). The slides were exposed to the initial three solutions (ethanol-containing) for five minutes each, and were exposed to the final two solutions (xylene) for two minutes per solution. The slides were manually mounted using Dako hard-set mounting media (Dako, cat# CS703), with unmounted slides left in the final xylene bath until mounting. Mounted slides were air-dried overnight at room temperature.

2.9.1.2. Imaging procedure

Slides were imaged using either an Olympus Virtual Slide Imager VS120 or an Axio Lab.A1 (Zeiss) microscope with attached AxioCam ICc5 (Zeiss). Images taken using the VS120 slide scanner utilised the bundled imaging software, while the AxioCam utilised Zen (Blue Edition, v 2.3).

The Olympus scanner was set to take an image of size 6mm x 6mm from each well at 20x objective magnification. The Axio Lab.A1 was used at 20x objective magnification to image 16 non-overlapping fields of view.

2.9.2. Converting image files to usable formats and size

The Olympus Virtual Slide Imager VS120 created all image files in a proprietary filetype (.vsi), which was not readable by most imaging programs. Although FIJI Is Just ImageJ (FIJI) could open these files using the Bioformats plugin, due to the size of the images (>1GB) it was nearly impossible to analyse the images using available equipment.

To aid in analysis, a macro was written to firstly open every image (around 700-750 images) and convert it into the .tiff file format, allowing the images to be opened easily by FIJI without the use of the Bioformats plugin (see Appendix 1: FIJI and ImageJ Macros (Raw Code) for macro codes). A second macro was then written to take each .tiff file, convert it into RGB colour (to allow analysis of staining, discussed in the next section) and then select 16 non-overlapping fields of view and save each field of view as a separate image. The fields of view were selected at fixed locations spread across the whole image and were positioned identically in every image, to prevent bias when selecting areas to analyse. This produced more than 11,000 images.

2.9.3. Image analysis

Image analysis of immunocytochemical stainingstaining was performed using a combination of ImageJ and FIJI. From each well (16 images total), five images were randomly selected by random number generator (using the website https://www.random.org/) and the nuclei in these images were manually counted using the point selection tool in ImageJ or FIJI.

Initially (for 9 slides – a total of 360 images) the staining in each cell was measured utilising the ImageJ plugin IHCToolbox [210]. However the scale of the analysis required automation. Dr. Jo-Maree Courtney wrote a macro for FIJI which randomly selected five images from each well and opened them sequentially without user input. The nuclei could then be counted manually for each image, following which the macro would apply colour deconvolution to the image. Colour deconvolution is an in-built FIJI function which can separate DAB and haematoxylin staining into individual channels, allowing the user to utilise the 'threshold' function of FIJI to select and measure the area stained with DAB without selecting haematoxylin-stained areas.

This macro was used to analyse the remaining images, although it required user-coded changes to accurately select the area stained and to ensure the proper scale was set for each image (see Appendix 1: FIJI and ImageJ Macros (Raw Code) for macro code). Data were expressed as area stained/cell, a measurement which was obtained by dividing the total area stained in an image by the number of nuclei present. Analysis of the data consisted of a two-way ANOVA applied to the three pHBEC groups treated with drugs, and all other data were analysed with one-way ANOVAs.

2.10. Enzyme linked immunoabsorbance assays

2.10.1. Collection of samples

2.10.1.1. Cell culture supernatant

Twenty four hours prior to collection of the cells the media was changed. The supernatant was decanted following twenty four hours (in the case of CSE treated cells the media was collected after 4 hours) and centrifuged at 1,500rpm for ten minutes at 4°C. Following centrifugation the supernatant was split into aliquots (1ml) being careful not to disturb the pellet and stored at -80°C.

2.10.1.2. Intra-cellular protein in PBS

Cells were grown in 12-well plates as described in section 2.4.1 for pHBECs and section 2.6.3 for BEAS-2B cells. RNA extraction was performed using the AllPrep RNA/Protein kit (QIAgen, cat# 80404), as according to the manufacturer's instructions. Briefly, the cells were washed with PBS and buffer APL (200µl) was added to each well and allowed to incubate at room temperature for five minutes. Following incubation the well bottom was scratched vigorously with the tip of a micropipette and the lysate transferred to an AllPrep spin column after being pipetted up and down several times in order to further help lysis. The homogenate was spun through the column at 8,500g (Heraeus Pico21 centrifuge). For the first 24 samples, protein extraction was performed first, however for all subsequent samples RNA extraction was performed first.

For protein extraction, the Protein Cleanup spin column was vortexed briefly and, after the cap was loosened slightly and the bottom snapped off, centrifuged for 3 minutes at 700g

(Heraeaus Pico21 centrifuge). PBS (500µl; Life Technologies, cat# 10010-023) was added to the spin column and then the column was centrifuged at 700g for 3 minutes. Protein-containing flow through was kept on ice prior to addition to the columns. The flow through from the AllPrep column was pipetted dropwise slowly onto the gel and then the sample was centrifuged at 200g for 3 minutes. The resultant flow through, which contained total protein, was stored at -80°C until use.

Note: *Analysis of intracellular N-cadherin protein was not possible due to readings being below the threshold of the standard curve.*

2.10.2. ELISA protocol

Pro-collagen 1-α DuoSet ELISAs (R&D Systems, cat# DY6220-05) were performed according to the manufacturer's instructions. Briefly, a standard curve was produced by diluting the provided standard protein 1:30 in PBS and then creating a series of dilutions (1:2) down to 39pg/ml. The detection antibody was diluted 1:10 to a concentration of 100ng/µl.

A 96-well plate was coated with capture antibody (4µg/ml, 100µl) overnight at room temperature. Wells were washed three times with wash buffer before the wells were protein-blocked by adding reagent diluent (300µl) to the wells for 1 hour at room temperature. Wells were washed three times with wash buffer.

Cell culture supernatant (collected as described in 2.10.1.1) or standard was added to the wells and incubated for 2 hours at room temperature. Wells were washed three times with wash buffer. Detection antibody (100µl) was applied to the wells for 2 hours at room temperature, after which the wells were washed three times with wash buffer. Streptavidin-HRP (100µl) was added to each well and incubated in the dark for 20 minutes at room temperature. Wells were washed three times with wash buffer.

Substrate solution (100µl) was applied to each well and incubated in the dark for 20 minutes, before stop solution (50µl) was added to each well. Plates were read using a Spectramax M2 (Molecular Devices) at 450nm (for quantification of sample) and 540nm and 570nm (for wavelength correction). All results were corrected using the average value obtained by the 540nm and 570nm readings.

, Analysis of the data consisted of a two-way ANOVA applied to the three pHBEC groups treated with drugs, and all other data were analysed with one-way ANOVAs.

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA – *long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

2.11. Statistical analyses

All mathematical calculations were performed in Microsoft Excel 2013 and statistical analysis was performed in Graphpad Prism (v 7.03) and all data were represented as the mean value with error bars showing standard error of the mean (SEM). Analysis of the multi-group data usually consisted of a two-way ANOVA applied, while all other data were analysed with one-way ANOVAs. For subsequent comparisons of two potential groups of difference, Welch's t-tests were performed for paired data, however where values were missing in paired data sets an unpaired t-test was performed instead. For the analysis of binomial data such as survival, Fisher' exact test was used.

2.12. Reagent and buffer composition appendix

2.12.1. Tris-HCl (0.5M) pH 7.6 with TWEEN20

Used in: 2.7.2 and 2.9.1

Ingredients

60.56g trishydroxy-methyl-amino-methane (Merck; cat# 1083822500)

90g NaCl

HCl (approx. 42ml)

5ml TWEEN20 (Sigma-Aldrich, cat# P-7949)

Method

1. Dissolve trishydroxy-methyl-amino-methane in 800ml de-ionised water.
2. Add NaCl and dissolve.
3. Adjust pH to 7.6 using HCl (and NaOH if needed).
4. Add TWEEN20 and make up to 1L in volumetric flask.
5. Check pH and adjust back to 7.6.
6. Make up to 10L with deionised water.

2.12.2. Primary antibody information

Used in: 2.7.2 and 2.9.1

All primary antibodies used were anti-human.

Table 2.12-1 Antibody information for biopsy staining. *Information for the primary antibodies used in section 2.7.2 for the staining of FFPE biopsies obtained from volunteers with and without COPD.*

KEY: * indicates that appropriate animal complete serum was used as the isotype control for the indicated antibody.

Antibody target	*Animal IgG*	*Clonality*	*IgG type*	*Supplier*	*Catalogue number*	*Dilution factor*
E-cadherin	Mouse	Monoclonal	1	Dako	M3612	1:100
N-cadherin	Mouse	Monoclonal	2a	Invitrogen	X18-0224	1:100
Pan-cytokeratin ae/3	Mouse	Monoclonal	1	Dako	M3515	1:100
Vimentin	Mouse	Monoclonal	1	Dako	M7020	1:2,000
S100A4	Rabbit	Polyclonal	*	Dako	A5114	1:3,000

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

Table 2.12-2: Antibody information for cell staining. *Information for the primary antibodies used in section 2.9.1 for the staining of cultured cells obtained from volunteers with and without COPD.*

Antibody target	*Animal IgG*	*Clonality*	*IgG type*	*Supplier*	*Catalogue number*	*Dilution factor*
E-cadherin	Mouse	Monoclonal	1	Dako	M3612	1:200
N-cadherin	Mouse	Monoclonal	2a	Invitrogen	X18-0224	1:200
Pan-cytokeratin ae/3	Mouse	Monoclonal	1	Dako	M3515	1:200
Vimentin	Mouse	Monoclonal	1	Dako	M7020	1:1,000
PAFR	Mouse	Monoclonal	1	Cayman Chemical	160600	1:200
ICAM-1	Mouse	Monoclonal	1	Chemicon	mab2130	1:200
TWIST	Mouse	Monoclonal	1	Abcam	ab175430	1:200

3. Comparative growth of primary bronchial epithelial cells cultured from volunteers with and without COPD and the effects of cryopreservation on primary cells

3.1. Introduction

Primary cell cultures are taken directly from the tissue of animals, patients or plants and cultured with the aim of maintaining their *in vivo* phenotype as much as possible. Primary cells are widely considered to provide a more accurate representation of *in vivo* disease phenotypes and mechanisms, especially considering that they can be isolated from patients with diseases. WhileWhile healthy cells can be induced to model the phenotype, since induction often only involves one or a few pathways whereas a disease cannot always be accurately artificially replicated *in vitro* as the condition is often the result of a myriad of interconnected signals and mechanisms which work together to contribute to the condition. This makes primary cells from diseased patients especially valuable as models.

However, cells taken from non-cancerous tissues have a finite lifespan, as they experience senescence and a decline in growth and proliferation [218, 219]. This is in comparison to cells lines and cells derived from cancer, which are 'immortalised' either through mutation or via transformation with a virus such as the SV40 adenovirus. Primary cells' limited lifespan is due to the fact that they can divide only a limited number of times before undergoing senescence; this limit is called the 'Hayflick limit' after the author who first described it, and is one of the major issues associated with culturing primary cells [222].

The Hayflick limit is not the only consideration when culturing primary cells. Simply isolating the desired cell type and maintaining it as a pure culture can be difficult. Since primary cells are sourced directly from the organ of interest, which is typically not formed of a single homogenous cell type, often the desired primary cells can be overtaken by more readily proliferating cells which are inadvertently collected alongside the target cells. In epithelial cultures, fibroblasts are particularly well known for overtaking cultures [223]. Even if attempts are made to physically isolate or dissect out the area of collection or to remove cells from a specific area, unwanted cells can still be collected and proliferate significantly before being noticed [221].

Contamination with other cell types from the tissue of origin is not the only source of cellular contamination primary cultures are exposed to. There are reports of cell cultures being overtaken by hardy, immortalised cells which are cultured in the same space, often entering cultures through shared media, and which can completely subsume a less proliferative primary culture [222-227]. Cross-contamination can occur even when the culture of the different cell types is separated in time [229], or even between cultures of different species [225].

An additional concern when collecting primary lung cells from people with diseases like COPD who often have *in vivo* chronic bacterial colonisation and carry a high overt burden of infection [48], is that pathogens may be collected along with the desired cells. Fungi and bacteria are common cell culture contaminants, and can be extremely resistant to attempts to deter their growth, particularly if they are innoculated directly into the cultures during collection. Additionally, diseased cells may be more susceptible to invading pathogens than

healthy cells, and therefore an important facet of the study may be irrevocably impaired if an infection manages to begin spreading among samples. Steps can be taken to reduce the risk of these contaminants, including refusing to sample patients who have had recent infections, however healthy lungs and airways are naturally colonised by bacteria [228-230], which may potentially contaminate cultures.

Finally, once primary cells have been successfully isolated and cultured, they are typicallyused immediately, without cryopreservation [33, 103, 111, 192, 194, 231-236]. Previous work has been done in isolating and culturing primary bronchial epithelial cells (pHBECs) from healthy and COPD-affected airways, however none have looked at cryopreservation of these valuable cells. Generally, primary bronchial epithelial cells have been obtained in a number of ways, ranging from the ex vivo culture of airway biopsies [33, 103, 231] to extraction from biopsied tissue via enzymatic isolation [111, 232, 235, 236], purchase from a commercial cell bank [194, 233, 234] or removal of the cells from the airway wall via brushing during bronchoscopy [192]. These cells were used directly following collection with only minimal culture time, although some cultures were passaged as high as passage 6 before use [189]. However, they are not cryopreserved as is common in immortalised cell lines. While immediate usage avoids the Hayflick limit and changes brought on by continual passaging, it presents some challenges. When cells are collected regularly, however do not proliferate rapidly enough to be used in experiments before more cells are collected the backlog of growing cells can quickly fill all available culture space. New samples may have to be refused due to lack of space and resources to culture them, which is undesirable when collecting such a limited resource as primary cells. Cryopreservation is commonly used to avoid such space-related issues in immortalised cells

and cancer-derived cell lines, as it allows cells to be frozen and stored until needed. This allows samples to be brought up in controlled amounts and used when needed. It also allows back-up cells to be kept safe against possible loss or experimental failure or for future experiments. However, this technique is not applied to primary bronchial epithelial cells, possibly due to concerns about the cells being too fragile to survive freezing.

There are significant advantages to utilising primary cells for modelling disease and other conditions, however it is important to keep the issues inherent in using primary cells in mind when culturing these cells. The process of cryopreserving these valuable cells and thus having more control over how and when they are used, as well as opening up the possibility of banking these cells for later study, is an important consideration, and the effects of the process of pHBECs is currently unknown. The current chapter of this thesis will explore the growth of primary epithelial cells isolated from the airways of non-smokers, smokers with normal lung function and people with airflow limitation who are either current or ex-smokers, via bronchial brushing. Of particular interest was whether the primary cells suffered deleterious effects from cryopreservation, such as retarded growth or reduced viability, ifif stored in liquid nitrogen for a period of between 2-24 months before use.

3.1.1. Chapter aims

The aims of this chapter were as follows:

- To establish stable, pure and proliferative epithelial cell cultures obtained via bronchial brushings from healthy non-smokers, smokers with normal lung function, smokers with airlflow limitation and ex-smokers with airflow limitation.
- To characterise these cells' growth and determine whether growth is affected by disease or smoking status.
- To examine the effect of cryopreservation upon the primary cells' growth and identify any potential differences due to smoking or disease status.

Hypothesis: Smoking and airflow limitation, both with and without smoking, will have a negative effect on primary bronchial epithelial cells' ability to survive and proliferate in a culture situation and will negatively affect the cells' ability to survive cryopreservation and to recover and proliferate following freezing.

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

3.2. Results

3.2.1. Phenotypic examination of primary bronchial epithelial cells in culture

Ofthe 50 volunteer samples collected, all volunteers which produced viable cultures produced at least one culture of epithelial cells; of 204 successful cultures, only three were overrun with fibroblasts. Primary cells were considered to be epithelial if they appeared to consistently exhibit standard epithelial 'cobblestone' morphology based on visual evaluation of morphology during culture. Cells taken from non-smokers typically showed a normal epithelial cobblestone morphology when sufficiently confluent (Figure 3.2-1 - top left). Some mildly fibroblastic cells were noted in cultures from smokers with normal lung function (Figure 3.2-1 - top right) and people with airflow limitation (Figure 3.2-1 - bottom left), however these did not match the elongated spindle-shape and organised growth pattern of cells seen in fibroblast overrun cultures (Figure 3.2-1 - bottom right). Despite these abnormal cells in cultures from smokers with normal lung function and people with airflow limitation, the sporadic nature of these cells across the entire sample (estimated as <10% of cells at the most prolific) and the fact that the majority of cells exhibited normal epithelial morphology meant that these cultures were deemed pure epithelial cultures.

Although no further investigation of epithelial characteristics was undertaken at this point in the study, later studies (see following chapters) observed the cells' expression of epithelial and mesenchymal markers and confirmed that these cells expressed epithelial markers such as E-cadherin and tight junction protein-1. Overall, bronchial epithelial cells were successfully isolated and cultured from volunteers both with and without airflow limitation,

and from people with different smoking statuses, with only minor contamination of fibroblasts resulting in culture loss.

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA – *long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

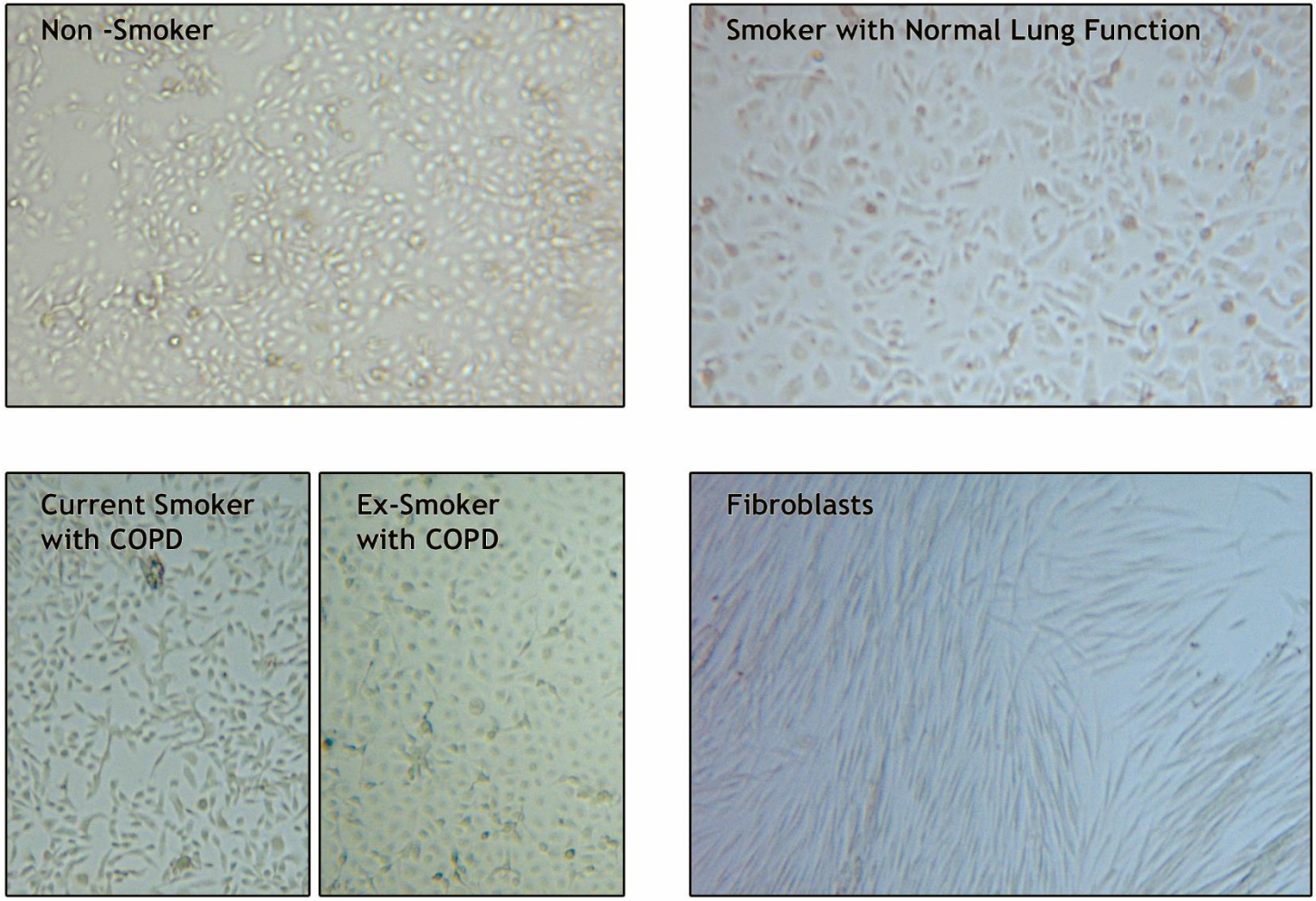

Figure 3.2-1: Morphology of primary epithelial cell cultures VS fibroblasts. *pHBECs were grown in submerged culture in BEGM following collection from volunteers, as described in the Methods and Materials section. Images were taken immediately prior to the first passage following collection using phase contrast microscopy at 4x objective magnification. Images presented here were representative of the entire culture.*

Please note: *image brightness and hue have been slightly adjusted for clarity.*

3.2.2. Rates of growth and survival of cells by smoking status after isolation

During the initial stages of culture, following removal of the cells from the airways until initial passaging, the survival rates between the four investigative groups was similar. Approximately 80% of all flasks reaching 80-90% confluence, allowing them to be passaged (Figure 3.2-2). Cells from current smokers with airflow limitation (COPD-CS on the graph) trended towards decreased survival, however this was not significantly different from the other groups (Fisher's exact test NNS versus COPD-CS $p = 0.32$, NLFS versus COPD-CS $p = 0.81$, COPD-CS versus COPD-ES $p = 0.35$). The survival data do not have error bars as the data are binary. Every flask which reached sufficient confluence to be passaged was classed as surviving; all other flasks were designated as not surviving.

Around 5-10% of flasks were discarded due to fungal infection, however this was a single infection that spread between samples at an isolated time. The infection was identified as fungus both by microscopic examination and by the large, attached 'fuzzy' islands of fungal growth that floated in the cultures. The origin of the fungus was likely improperly processed water placed into the incubator's humidifying tray, rather than contamination originating from a sample, as multiple samples exhibited superficially identical fungal growth at the same time. The fungus persisted and proliferated despite anti-fungal compounds in the culture media, but was eradicated following a move of all non-affected flasks into a different incubator and disposal of infected flasks. The incubator was subsequently sterilised and infection did not recur. Aside from cultures which became infected with fungus, no volunteer sample failed to yield at least one flask which reached confluence, allowing passaging to occur.

The average time taken for the first flask within a given volunteer sample to reach confluence was approximately 15-20 days (Figure 3.2-3) although there was a large degree of variability between patient samples, and even within a single sample there could be up to five days of difference between the first and last flask passaged.

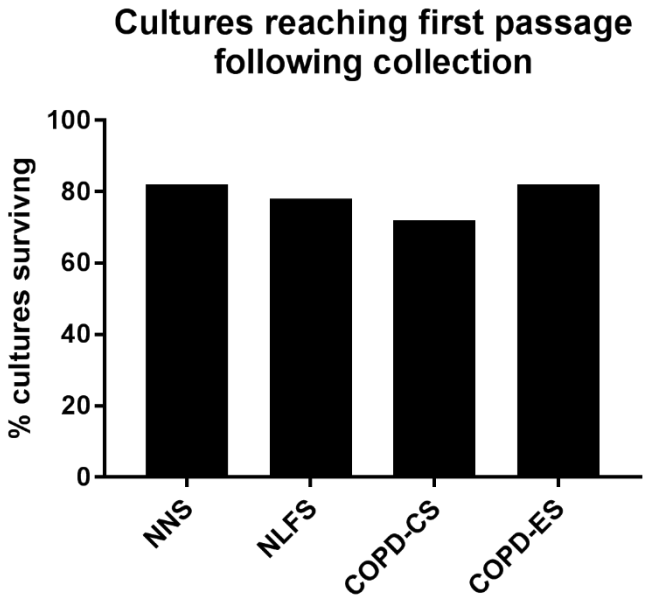

Figure 3.2-2: Survival rates for primary cells following collection. *Each volunteer provided four individual brushings from the airways, each one producing a separate culture. Additionally, the four brushes together provided an additional culture, meaning each volunteer produced 5 flasks in total. Cells were grown in submerged culture in BEGM until 80-90% confluent and were then passaged. Failed samples did not reach confluence, either due to lack of proliferation, death of cells or infection.*

KEY: NNS = non-smokers; NLFS = smokers with normal lung function; COPD-CS = current smokers with COPD; COPD-ES = ex-smokers with COPD.

n = NNS: 78; NLFS: 46; COPD-CS: 32; COPD-ES: 45

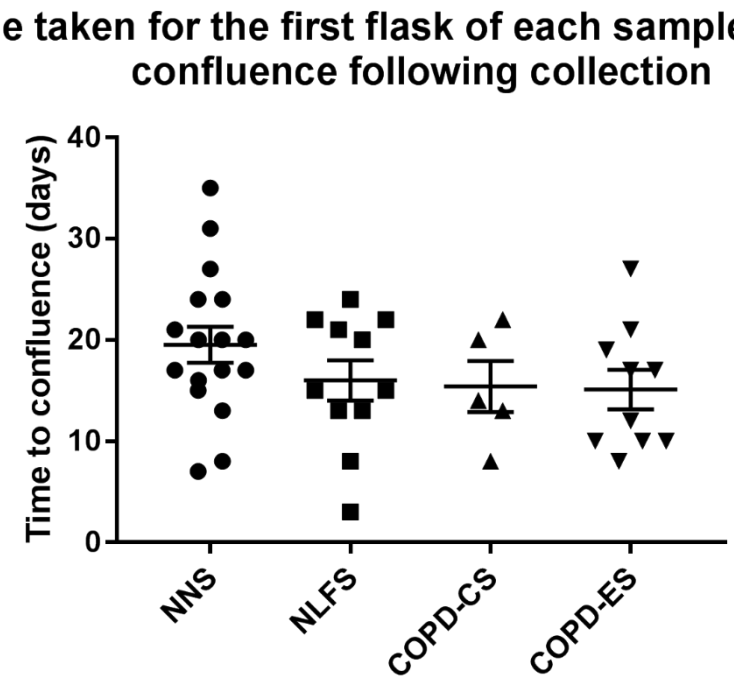

Figure 3.2-3: Time between collection and first passage for primary bronchial epithelial cells. *Each volunteer provided four individual brushings from the airways, each one producing a separate culture. Additionally, the four brushes together provided an additional culture, meaning each volunteer produced 5 flasks in total. However, only the first culture to reach confluency for each volunteer was included here. Cultures from a single volunteer usually reached confluence within 3 days of the first culture. Cells were grown in submerged culture in BEGM until 80-90% confluent and were then passaged. Failed samples did not reach confluence, either due to lack of proliferation, death of cells or infection.*

KEY: NNS = non-smokers; NLFS = smokers with normal lung function; COPD-CS = current smokers with COPD; COPD-ES = ex-smokers with COPD.

n = NNS: 17; NLFS: 11; COPD-CS: 5; COPD-ES: 10.

3.2.3. Rates of growth and survival of cells by smoking status following first subculture

Following the initial passage, samples from smokers with normal lung function exhibited a decrease in survival, from nearly 80% to 60% (Figure 3.2-4), which was significantly lower than the survival rates for non-smokers and ex-smokers with airflow limitation (Fisher's exact test NNS versus NLFS $p = 0.0058$, NLFS versus COPD-ES $p = 0.0052$). However, it was not statistically significantly different from the survival rates of samples for volunteers with airflow limitation who were current smokers (Fisher's exact test $p = 0.14$). A single volunteer sample was lost to fungal infection during this period of growth. The infection was caused by spread of a single fungal infection (see section 3.2.1), contaminating flasks which were passaged during the fungal infection period, with all flasks within that sample being contaminated during passaging.

During passage 1 cells took an average of 13-19 days to reach sufficient confluence to be passaged (Figure 3.2-5). p

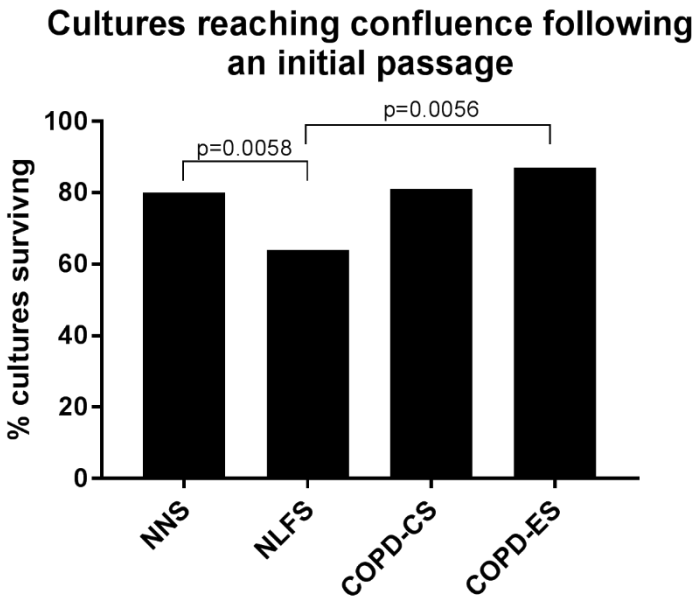

Figure 3.2-4: Survival rate of cells following initial passage. *Each culture which successfully reached confluence after the initial subculture was enzymatically dissociated and cryopreserved, stored in vapour phase liquid nitrogen. Cells were grown in submerged culture in BEGM until 80-90% confluent and were then cryopreserved. Failed samples did not reach confluence, either due to lack of proliferation, death of cells or infection.*

KEY: NNS = non-smokers; NLFS = smokers with normal lung function; COPD-CS = current smokers with COPD; COPD-ES = ex-smokers with COPD.

n = NNS: 107; NLFS: 53; COPD-CS: 32; COPD-ES: 67

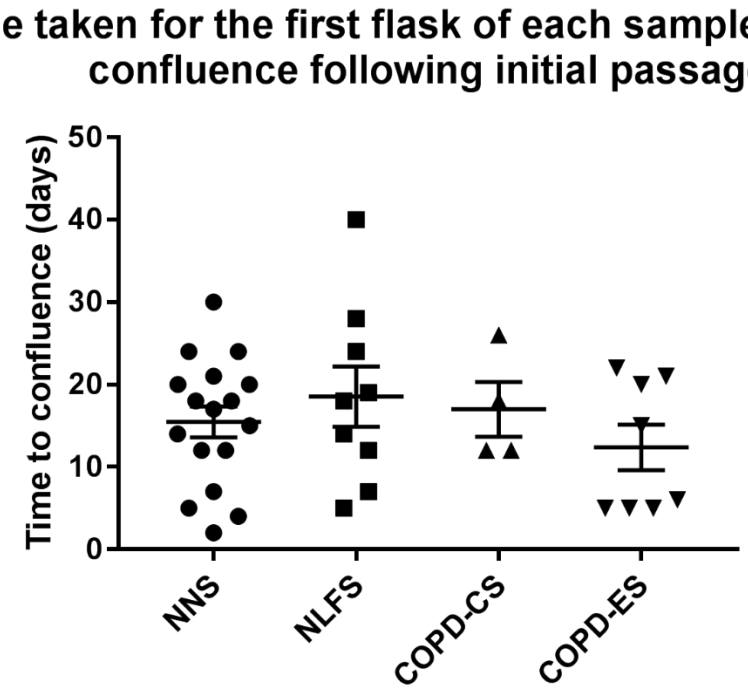

Figure 3.2-5: Time between first passage and cryopreservation for primary bronchial epithelial cells. *Each culture which successfully reached confluence after the initial subculture was enzymatically dissociated and cryopreserved, stored in vapour phase liquid nitrogen. Cells were grown in submerged culture in BEGM until 80-90% confluent and were then cryopreserved. However, only the first culture to reach confluency for each volunteer was included here. Cultures from a single volunteer usually reached confluence within 3 days of the first culture. Failed samples did not reach confluence, either due to lack of proliferation, death of cells or infection.*

KEY: NNS = non-smokers; NLFS = smokers with normal lung function; COPD-CS = current smokers with COPD; COPD-ES = ex-smokers with COPD; p0 = passage zero; p1 = passage one.

n = NNS: 17; NLFS: 9; COPD-CS: 4; COPD-ES: 8.

3.2.4. Rates of growth and survival of cells by smoking status after cryopreservation

Bronchial epithelial cells from both non-smokers and ex-smokers with airflow limitation had a survival rate of almost 60% when brought up from frozen stocks (Figure 3.2-6). In comparison, cells from current smokers with airflow limitation trended towards decreased survival, with only 40% of samples surviving. The trend was even more pronounced in cells from smokers without airflow limitation survived only 27% of the time, although due to small sample sizes the trends were not statistically significant. Previously three vials of cells had been harvested and cryopreserved from a single confluent T-755 flask at passage 1, and initially a single vial was used to seed a single T-75 flask at low seeding density. However, the low seeding density caused low survival rates and poor proliferation, which prompted changes in methodology; with cells being seeded at high density and used after seven days. Only cells seeded at the low density were included in the data, as the highly-seeded cells were used regardless of growth.

In the time taken for cells to proliferate sufficiently to be used in experiments following cryopreservation and thawing, there was no difference between cells taken from non-smokers and ex-smokers with airflow limitation (Figure 3.2-7). Commentcould not be made on smokers with normal lung function (n=1) or current smokers with airflow limitation (n=2), other than to say that anecdotally, they grew more consistently close to the average for ex-smokers with airflow limitation than the non-smokers. However, this should not be taken as a serious consideration, given the large variation seen in the non-smokers' time of growth. There waswas no statistically significant difference between the times of growth for non-

smokers and ex-smokers with airflow limitation, although the latter demonstrated a more precise spread with considerably lower variation than the non-smokers.

It is important to note that Figure 3.2-6 and Figure 3.2-7 contain combined data from myself and Kaitlyn Scoyler, who also worked with these cells.

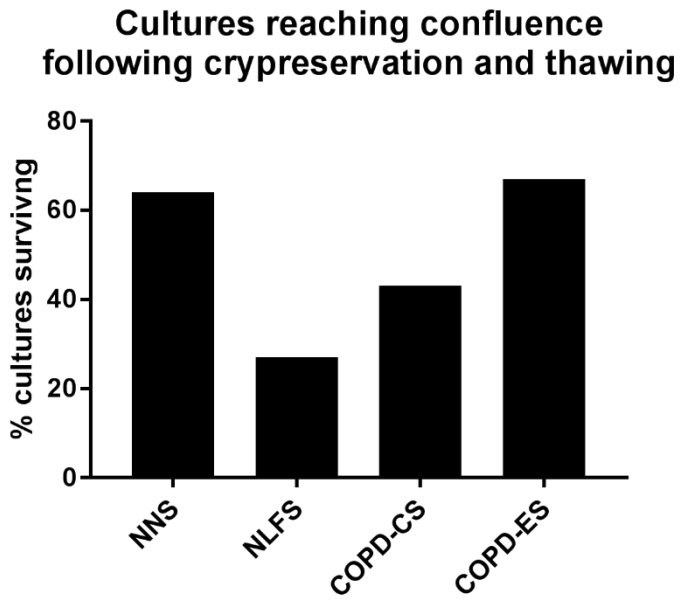

Figure 3.2-6: Survival rates for primary cells brought up from cryopreservation. *Flask survival following cryopreservation dropped to approximately 60% for non-smokers (NNS) and ex-smokers with COPD (COPD-ES). Each culture which successfully reached confluence after the initial subculture was enzymatically dissociated and cryopreserved, stored in vapour phase liquid nitrogen. Cells were grown in submerged culture in BEGM until 80-90% confluent and were then cryopreserved. Failed samples did not reach confluence, either due to lack of proliferation, death of cells or infection.*

KEY: NNS = non-smokers; NLFS = smokers with normal lung function; COPD-CS = current smokers with COPD; COPD-ES = ex-smokers with COPD.

n = NNS: 11; NLFS: 15; COPD-CS: 7; COPD-ES: 9.

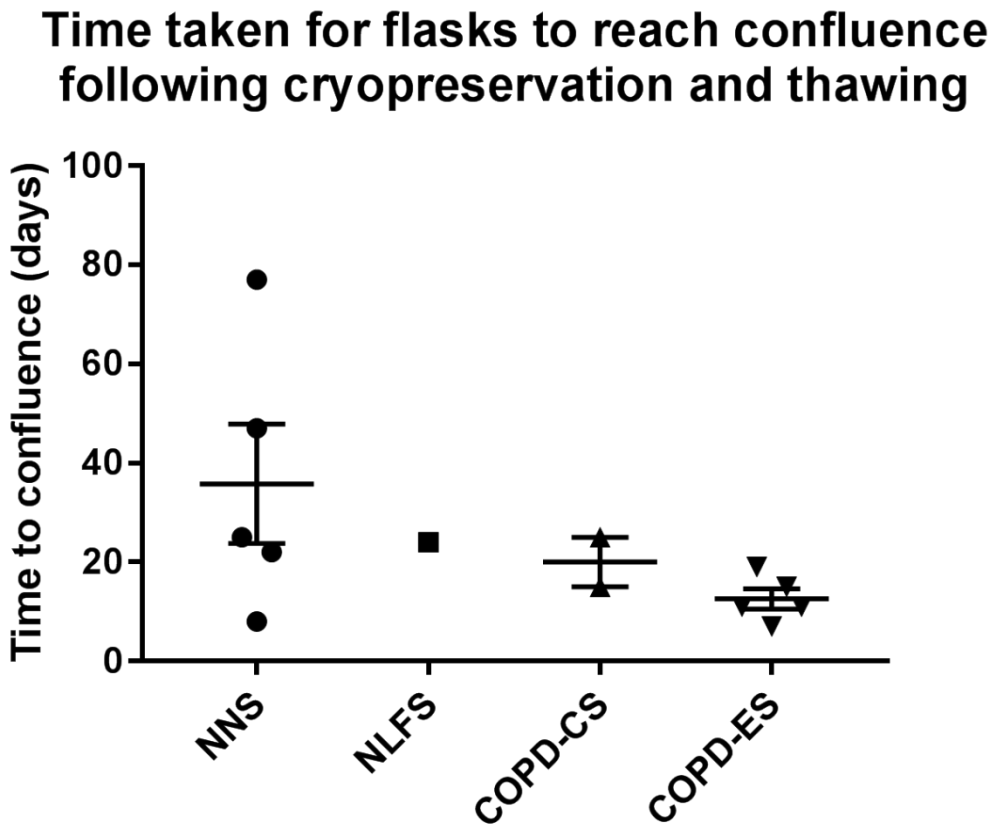

Figure 3.2-7: Time taken for samples revived from cryopreservation to reach levels of growth which were sufficient for samples to be collected. *Each culture which successfully reached confluence after the initial subculture was enzymatically dissociated and cryopreserved, stored in vapour phase liquid nitrogen. Cells were grown in submerged culture in BEGM until 80-90% confluent and were then cryopreserved. However, only the first culture to reach confluency for each volunteer was included here. Cultures from a single volunteer usually reached confluence within 3 days of the first culture. Failed samples did not reach confluence, either due to lack of proliferation, death of cells or infection.*

KEY: NNS = non-smokers; NLFS = smokers with normal lung function; COPD-CS = current smokers with COPD; COPD-ES = ex-smokers with COPD; p2 = passage two; p3 = passage three.

n = NNS: 5, NLFS: 1; COPD-CS: 2; COPD-ES: 5.

3.2.5. Effect passaging and cryopreservation on primary cells by smoking status

Comparing the survival rates within each group and considered across all passages, trends occur (Figure 3.2-8). Non-smokers did not appear to be affected by passaging alone, and while there was a 15% decrease in survival rate following cryopreservation, it did not reach statistical significance. However, given the apparent effect of cryopreservation on all the groups and based on the effects of passaging alone, it seemed likely that cryopreservation itself had a deleterious effect on cell survival. Passaging generally appeared to negatively affect the survival of cells taken from smokers with normal lung function. Although the changes only reached statistical significance following cryopreservation (Fisher's exact test p0-p2 $p = 0.0054$; p1-p2 $p = 0.0067$), there appeared to be a downwards trend at p1 which was intermediate between survival at p0 and survival following cryopreservation, but which did not reach statistical significance. Survial of cells from bothb current and ex-smokers with airflow limitation declined following cryopreservation, with current smokers' decreased survival reaching statistical significance, dropping by nearly 30% (Fisher's exact test $p = 0.0399$), suggesting a likely smoking effect.

Despite the changes in survival rates, smokers with normal lung function and current and ex-smokers with airflow limitation exhibited no change in their rate of growth as passaging progressed, averaging around 15-17 days to reach confluency (Figure 3.2-9). Only non-smokers showed any change in their growth rate, increasing from 15 days at passage 1 to 35 days following cryopreservation (ANOVA p0-p2 $p = 0.022$; p1-p2 $p = 0.036$).

Tracking a select few samples individually across passages in an attempt to discern the effects of cell density on the rate of growth following collection and the effect of

cryopreservation at an individual level rather than as a whole sample group (data not shown) revealed that cultures which took fewer than thirty days to reach confluence following collection generally took approximately the same amount of time to reach confluence following passaging, whereas those that took more than thirty days may have taken so long due to low cell seeding density, as passaging appeared to lower the time taken to reach confluence to levels comparable to the other samples. The only exception is in current smokers with airflow limitation, where the firstfirst passage appeared to cause a uniform, albeit slight, increase in time taken to reach confluence. Cryopreservation typically appeared to increase the time taken for cells to reach confluence.

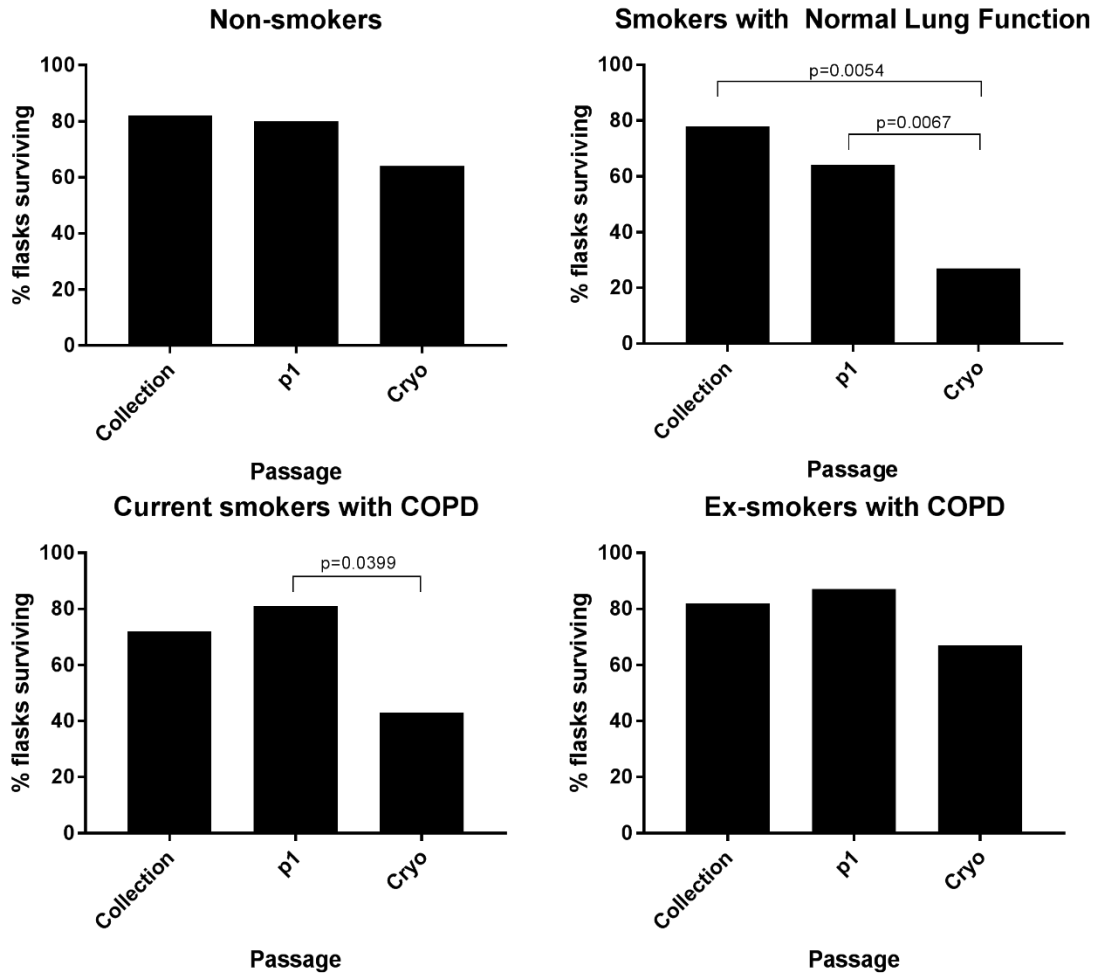

Figure 3.2-8 Comparison of survival rates in the phenotypical groups following subdivision and cryopreservation. *Cells were grown in submerged culture in BEGM until 80-90% confluent and were then sub-divided following p0 or cryopreserved following p1. Cells in the 'cryo' group were brought up from frozen. Failed samples did not reach confluence, either due to lack of proliferation, death of cells or infection.*

KEY: NNS = non-smokers; NLFS = smokers with normal lung function; COPD-CS = current smokers with COPD; COPD-ES = ex-smokers with COPD; p1 = passage one; cryo = post-cryopreservation.

n = NNS: 11-107; NLFS: 15-53; COPD-CS: 7-32; COPD-ES: 9-67

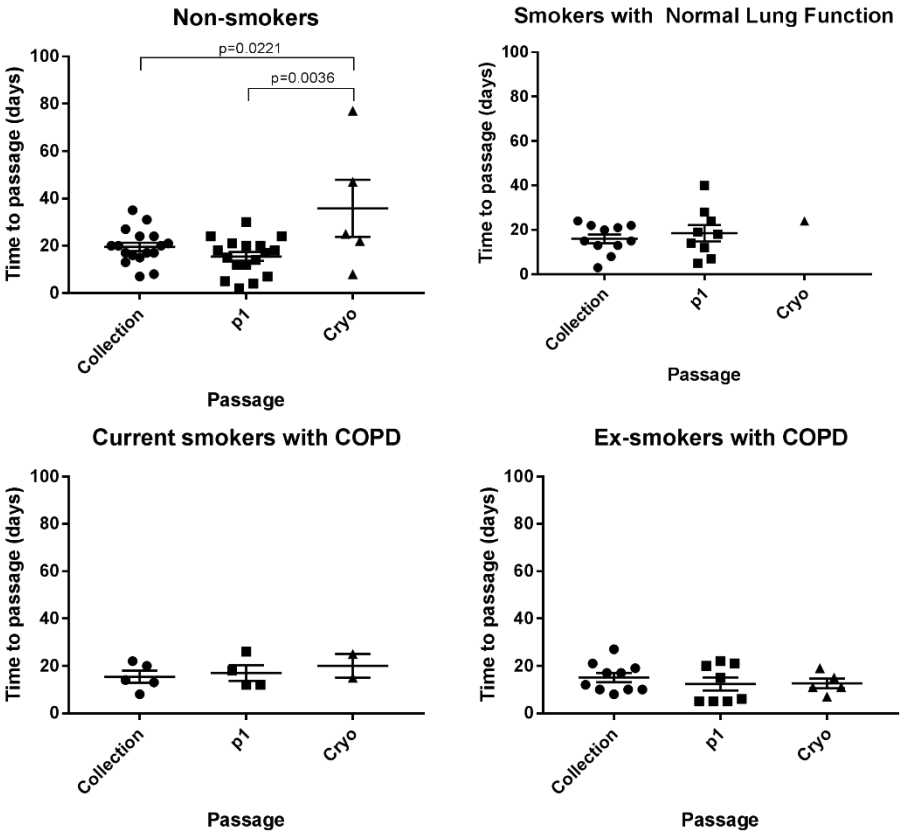

Figure 3.2-9: Changes in the length of time taken for samples in the four phenotypical groups to grow to confluence following subdivision and cryopreservation. *Cells were grown in submerged culture in BEGM until 80-90% confluent and were then sub-divided following p0 or cryopreserved following p1. Cells in the 'cryo' group were brought up from frozen. However, only the first culture to reach confluency for each volunteer was included here. Cultures from a single volunteer usually reached confluence within 3 days of the first culture. Failed samples did not reach confluence, either due to lack of proliferation, death of cells or infection.*

KEY: NNS = non-smokers; NLFS = smokers with normal lung function; COPD-CS = current smokers with COPD; COPD-ES = ex-smokers with COPD; p1 = passage one; cryo = post-cryopreservation.

n = NNS: 5-17; NLFS: 1-11; COPD-CS: 2-5; COPD-ES: 5-10.

3.4. Chapter discussion

Primary bronchial epithelial cells were successfully cultured from airway brushings taken from non-smokers, smokers with normal lung function and people with chronic airflow limitation, whichwhich has been referred to as 'COPD' within figures in this thesis. These epithelial cells were successfully cryopreserved and brought back from frozen stock, however cells from current smokers, regardless of airflow limitation, exhibited decreased viability following cryopreservation.

This study collected cells via bronchial brushings partly due to agreement with hospital staff and partly due to the relative non-invasiveness compared to biopsy sampling, which is an established technique [192]. It was also thought that using brushings would reduce the possibility of the epithelial cultures becoming overrun with invasive fibroblasts from the airway wall and parenchymal, which is a risk when isolating primary cells, and fibroblast contamination affected only 3 out of 204 cultures in total.

Cells from smokers and people with airflow obstruction exhibited star-shaped, fibroblast-like morphology more frequently than cells from non-smokers and were often observed to 'clump' and form multi-cell layered clusters more frequently than the neatly cobblestone-like non-smokers' cells. Despite this, they did not take on the narrow, spindle-like shape of true fibroblasts, nor exhibit the organised flowing patterns of growth seen in the overrun flasks (Figure 3.2-1) and were determined to be merely slightly abnormal epithelial cells.

Primary cell lines are often not passaged extensively, due primarily to their known finite lifespans and fragility [222] as well as due to fear that excessive passaging may lead to a loss of their useful *in vivo* phenotype. Primary cells in research are often used without any

cryopreservation, although they may be passaged up to six times [33, 103, 111, 192, 194, 231-236]. Cryopreservation of cells is a well-established and common technique in immortalised cell lines. It is very useful, as it allows cells to be stored with no upkeep and using minimal space (a single liquid nitrogen storage container can hold many thousands of vials) and they can be stored for many years without the changes associated with long-term culture.

Passaging alone did not appear to have any significant effect on the growth rate of the samples. Non-smokers, which took an average of 20 days to reach confluence following collection, dropped to the same levels as the other groups, and all four groups averaged around 16 days to reach confluence following the initial passage (Figure 3.2-5). At no point prior to cryopreservation did the different groups show a significant difference in growth rates.

Although the growth rate of the pHBECs was unaffected by passaging, the same could not be said for their survival. Even at baseline, immediately following collection, cells from current smokers with airflow limitation were 10% less likely to survive than cells from the other groups, although the difference did not reach statistical significance (Figure 3.2-2). Passaging had a marked effect on the survival of the cells, with only 60% of current smokers with normal lung function's flasks surviving, compared to 80% for the other groups (Figure 3.2-4). The decline was not statistically significant when compared to the cells' survival at passage 0. The fact that there was no significant difference between the survival rate of the smokers with normal lung function and that of current smokers with airflow limitation suggested that this decline was probably a smoking-related issue.

Interestingly, there did not appear to be an airflow limitation-related effect at the initial stages of culture following collection, since neither current nor ex-smokers with airflow limitation showed significant changes in their survival compared to non-smokers, despite the literature suggesting that cells taken from people with COPD are more likely to die than those from non-smokers [30]. However, the difference between the literature and the observed results at this point may simply be due to the early stage of disease, and if cultures from people with clinically diagnosed COPD were taken, the results may have been closer to what was seen in the literature.

It was not until cryopreservation following passage 1 that there were any significant changes to the growth rates of the cells. Due to insufficient numbers, objective comment could not be made on the cells from smokers with current lung function, nor on current smokers with airway obstruction. However cells from non-smokers took 38 days to reach confluence following cryopreservation, significantly longer than following collection or the initial passage (Figure 3.2-9). This demonstrated that although passaging has the potential to affect the growth of cells, cryopreservation of primary cells has a more immediate effect on the cells' ability to proliferate. In particular, pHBECs taken from healthy non-smokers were susceptible to the deleterious effects of cryopreservation, possibly because they were not normally subjected to harsh environmental stimuli (ie. cigarette smoke) and were thus less able to adapt rapidly to changing circumstances.

Cryopreservation significantly impacted the survival of cells taken from current smokers. Non-smokers' cells and cells from ex-smokers with airflow limitation survival dropped to around 60%, down 20% from previous levels. However, current smokers with airflow

limitation survived only 40% of the time and cells from smokers with normal lung function had a survival rate of only 26% (Figure 3.2-6). Despite the large differences in survival rates between the four groups, there was no statistical significance between groups, likely due to type 2 error caused by small sample sizes. However, looking within each group and tracking survival, the difference between cells from smokers with normal lung function before and after cryopreservation was significant, both when comparing post-collection and post-cryopreservation and passage 1 and post-cryopreservation survival rates (Figure 3.2-8). Cells from current smokers with airflow limitation also demonstrated a significant decline in survival following cryopreservation. Cryopreservation in general caused noticeable but non-statistically significant drop in survival across all groups, but cells taken from current smokers were less likely to survive than cells from any other group, whether considering the effect of passaging or cryopreservation. This study was not designed or equipped to investigate this interesting result, however it may be of importance in understanding the more acute effects of smoking, and quitting, on the airway epithelium. It is possible that the Nrf-2 and DAMP signalling pathways may be involved, as they have been implicated in cigarette smoke induced apoptosis and cell death in the airway epithelium in a number of studies [240-242]. It would be interesting to examine their interaction and any association between these pathways and the cells' survival following cryopreservation. Additionally, inhibition of the Nrf-2/DAMP pathways or blocking of pathway inhibitors might increase cell survival following cryopreservation, allowing more work to be performed on this valuable sample group.

Based on these results, while it is tempting to suggest that cryopreservation is not recommended for primary cells, the time which these cells were stored for (around two years)

likely played a role in the decline in survival noted here. It has previously been shown that the longer cells are stored, the fewer cells survive thawing [243], so while it is important to know that cells from smokers with normal lung function suffer more from freezing than other cells, if cryopreservation was used for short-term storage, it may alleviate some of the effect. The cells may also have been suffering from the effects of the Hayflick limit, as the cells were encouraged to proliferate greatly before storage (See Materials and Methods 2.4.1 for more details).

Overall, passaging and cryopreservation were detrimental to pHBECs' survival, however they affect cells differently depending on the cells' source. Although the effects of passaging vs cryopreservation cannot be fully untangled within this study, the fact that in the literature primary cells can survive for more than three passages suggests the effects seen here may be primarily attributed to cryopreservation, possibly with some interference from the Hayflick limit. This may be due to insufficient levels of cryoprotectant (DMSO), the cells may require additional supplementation with serum in their media immediately following thawing, or it may simply be an unavoidable side-effect of freezing primary cells. However, a major factor is the fact that the cells were stored for two years prior to use, as prolonged storage is known to affect the viability of cells [243]. Further work, with samples collected both before and after cryopreservation for molecular study, as well as samples which were passaged without freezing paired with frozen samples would help to understand the effects of the cryopreservation process on these valuable cells. Additional work to reduce the impact of multi-year storage would also be valuable, and when working with cells taken from current smokers with normal lung function, special care is required to ensure their survival.

3.5. Chapter conclusions

At early stages it appeared that neither passaging nor cell seeding density at collection had any significant effect on the rate of growth of primary cells taken from non-smokers, smokers with normal lung function or people with airflow limitation, regardless of current smoking or disease status. However, cryopreservation negatively affected cells from non-smokers, nearly halving their growth rates, yet had no effect on ex-smokers with airflow limitation.

Smoking appeared to negatively affect the survival rate of cells, with cells from current smokers surviving less often than those from non- or ex-smokers. Although initially cells from all groups survived about 80% of the time, after only one passage, cells taken from smokers with normal lung function had a lowered survival rate compared to the other groups, falling to around 60%. Cryopreservation severely impacted these cells' survival – although the non-smoking groups suffered from a 10% decrease in survival after cryopreservation, cultures from smokers with airflow limitation showed a 30% decreased survival rate compared to earlier passages, with only 40% of cultures surviving. Likewise, current smokers with normal lung function exhibited decreased viability after cryopreservation, with a survival rate after cryopreservation of only 26%, a drop of nearly 60% from initial survival and 30% from passage 1. These results demonstrated that while cryopreservation is possible for pHBECs, it affects different groups of cells in different ways, and that cells from current smokers may be particularly sensitive to cryopreservation. It is advisable to attempt further optimisation of the process to reduce the deleterious effects of cryopreservation, possibly by altering levels of cryoprotectant or increasing cell density when bringing up frozen cells.

4. Modelling bronchial epithelial cells *in vitro* using primary cells and an immortalised cell line

4.1. Introduction

4.1.1. Changes in the epithelium in the large airways during COPD

The pseudostratified epithelial layer in the healthy, normal large airways provides both a barrier to foreign particles and bacteria, but also plays a key role in clearing particulates and debris from the lungs [19, 238]. The epithelial cells in the large airways possess cilia on their apical surface, which beat in time in order to move mucus and particles up the airways and out into the trachea, where they can be swallowed or otherwise expelled from the body [19]. In order to facilitate this, and to trap pathogens and unwanted substances, the epithelial layer has numerous goblet cells and sub-mucosal glands, and the mucus these cells and glands produce encapsulates pathogens and particulates, allowing them to move easily along the airway. Typically there is a minimal immune presence in both the airway walls and the lumen, as the airway epithelium is considered to be a site of 'immune privilege', similar to the testes [245]. However, in COPD, the epithelial layer is dysregulated and is the site of a number of pathological changes.

Squamous metaplasia, the thickening of the airway epithelium, is among the easiest COPD-realted change to spot, as is the proliferation and hyperactivity of mucus producing goblet cells and sub-mucosal glands [20-23]. There is also a decrease in cilia length and a corresponding reduction in airway clearance [24, 25]. Many of the more characteristic changes of the airway, however, such as immune cell activation and airway wall thickening,

occur in either the lumen or beneath the epithelial layer. It was not until 2010 that Sohal and colleagues proposed that the airway epithelium may be playing a different, more active role in COPD pathology, via the process of EMT [31]. Since 2010, it hass been demonstrated that the airway epithelium, particularly the basal cell layer, is highly dysregulated [26-30], expressing a number of mesenchymal markers such as S100A4, vimentin and α-smooth muscle actin [31-34], in addition to being highly activated via pathways such as the TGF-β related Smad pathway [207, 240, 241] and the Wnt signalling pathway [242]. Furthermore, the epithelial layer overexpresses viral and bacterial adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and platelet activating factor receptors (PAFr) [243-252]. Not only is the epithelium dysregulated *in vivo*, but it has also been demonstrated that epithelial cells taken from people with airflow limitation exhibit sustained pathological differences from cells taken from non-smokers.

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

4.1.2. Differences between epithelial cells isolated from non-smokers and people with airflow limitation *in vitro*

In vitro, epithelial cells taken from the airways of people with COPD do not proliferate as rapidly as those taken from non-smokers, nor do they survive and differentiate as well as cells from non-smokers when grown at an air-liquid interface (ALI) [26]. In 2010 Milara and colleagues were able to show that primary epithelial cells taken from the bronchi of people with COPD exhibited a sustained EMT phenotype in culture [33]. Fibroblasts taken from the airways of people with COPD have also shown that they secrete currently unknown factors into the surrounding environment which can cause epithelial cells to undergo EMT [34]. This suggests that bronchial epithelial cells taken from COPD airways exhibit a changed phenotype which endures in culture, potentially making them a valuable resource for identifying pathways and mechanisms.

In vitro cell models need to mimic the *in vivo* disease as closely as possible, and ALI cultures are considered good for examining the airway epithelium since they allow differentiation of the cells into a full epithelial layer with secretory and ciliated cells [26]. However, there is evidence that epithelial cell dysregulation in COPD begins and stems primarily from issues with the basal progenitor cells in the epithelium, which are not terminally differentiated and comprise about 50% of the epithelial cell population in a pseduostratified layer [253]. TraditionalT submerged culture does not allow a differentiated epithelium to form, instead causing proliferation of undifferentiated basal cells, making it potentially more useful for studying airway epithelial dysregulation in the sub-population of cells it affectsa most than ALI culture. In light of this, it is important to not dismiss submerged culture, even though it

does not produce a differentiated airway epithelium. However, it is crucial to check, if possible, that a culture model accurately reflects both the disease and the non-pathogenic state which is seen *in vivo*, since an inaccurate model is of no use to anyone.

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4.1.3. Biopsies and cell culture: how do they complement each other in research?

Biopsies and other *ex-vivo* fixed tissue samples such as whole organ excisions are a valuable resource in the understanding of normal organ function, and the pathological changes which occur in disease. They provide snapshots of a certain time and location in the disease, and provided they are stored correctly can be analysed many years in the future. They also allow researchers to look at the tissue as a whole, rather than as isolated cell types, revealing changes in structural relationships between different cell layers and structures. Unlike cell culture, these samples do not have the same risk of changes over time; they are not continuously growing and they are an accurate representation of the tissue at the time of collection. However, biopsies and other fixed samples can be negatively affected by the fixation process, which can produce artefacts, destroy or alter certain markers or types of molecule and, depending on the method chosen, render the tissue unsuitable for certain analyses. For example, the commonly used fixation solution paraformaldehyde forms strong cross-links between proteins in the cell which then require reversal using antigen retrieval [260], and even then some proteins are too altered or damaged to be stained. In addition to molecular level changes, structural changes are also a product of collection and fixation; for example, lumen may collapse following collection, and this makes studying structures such as airways and alveoli challenging. Finally, although they do not change over time in the same way that cells do, prolonged storage may degrade antigen epitopes and other biopsy features. Despite these challenges, biopsies provide key insights into disease, and can often provide a starting point for identifying interesting processes and molecular changes which can direct and be further elucidated by cell culture studies.

Compared to biopsies, cell culture is extremely versatile, but at the cost of sacrificing a certain amount of *in vivo* accuracy. As previously mentioned (section 3.1) cells undergo changes during prolonged culture, which can reduce their relevance to the disease in question. They also are isolated from the intercellular milieu of the body, and therefore may not accurately represent the full spectrum of disease, nor is it possible to ascertain if there are any knock-on effects in other important functional areas from seemingly minor changes in protein expression without complicated co-cultures.

However, the continual growth and proliferation of cell culture is also a boon – because they are still alive, the cells can be treated with drugs, cytokines or other molecules or compounds of interest, such as cigarette smoke, and their reactions observed. It is possible to treat cells with chemical substances it would be extremely difficult to administer to, or even get ethical approval to use on, humans. Cigarette smoke, and its effect on non-smokers' cells, is a prime example, and the effects of drugs can be tested without concern for potential systemic side-effects.

Together, biopsies and cell culture can complement each other well, with biopsies providingproviding information on the gross physiological disease state, and cell culture providing theoretically free-rein on manipulation of micro-conditions and detailed investigation and manipulation of pathways. It is very important to keep both sides in mind when researching processes such as EMT, and the comparison of culture with biopsy can potentially reveal if the cells truly represent the condition naturally seen in the organ of interest. In this vein, the main focus of this chapter is the question of how well primary cells taken from the airway epithelium maintain their phenotype in culture, and if the

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commercially available BEAS-2B cell line is truly an accurate representation of healthy primary bronchial epithelial cells with reagards to the process of EMT.

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4.1.4. Chapter aims

The aims of this chapter were as follows:

- To establish the baseline differences between the epithelium in biopsies taken from non-smokers, smokers with normal lung function and people with airflow limitation with regards to markers of epithelial-mesenchymal transition (EMT).
- To confirm that cultured cells taken from non-smokers, smokers with normal lung function and people with airflow limitation maintain their disease phenotype in culture by comparing their pattern of expression to the biopsies with regards to markers of epithelial-mesenchymal transition (EMT).
- To further compare cells taken from non-smokers, smokers with normal lung function and people with airflow limitationlimitation, looking at the expression of additional EMT markers, two signalling molecules and two extracellular matrix remodelling factors.
- To evaluate the suitability of the commercially available, immortalised BEAS-2B cell line as a model for healthy bronchial epithelial cells with regards to markers of EMT, two signalling molecules and two extracellular matrix remodelling factors.

Hypothesis: Airflow limitation will be marked by increased EMT signals and increased expression of signalling molecules and extracellular matrix remodelling proteins compared to bronchial epithelial cells from non-smokers. Bronchial epithelial, while cells from smokers with normal lung function will exhibit an intermediate level of EMT, although and all

cultured cells will exhibit a slightly decreased intensity of EMT signals following culture and cryopreservation compared to the biopsies. BEAS-2B cells will express similar levels of EMT-related markers as primary bronchial epithelial cells isolated from non-smokers.

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4.2. Results

4.2.1. Epithelial markers

The expression of E-cadherin in cultured primary cells matched the pattern of expression seen in the epithelium of the biopsies taken from non-smokers, smokers with normal lung function and people with airflow limitation, with no differences in expression being observed between groups (Figure 4.2-1). thisThe immortalised BEAS-2B cell line demonstrated a trend towards lower levels of E-cadherin expression than primary cells from non-smokers, although this was not statistically significant.

SimilarSimilar to E-cadherin, while the overall pattern of expression of cytokeratin between the three groups of primary cells matched the pattern observed in biopsies, there was no difference between the three primary cells groups, nor between primary non-smokers' cells and BEAS-2B cells (Figure 4.2-2).

While tight junction protein-1 (TJP1) expression was not measured in biopsies, its level of expression across all three groups of primary cells matched that seen in the other two epithelial markers, all three groups expressing the same level of TJP1 (Figure 4.2-3). However, unlike the other epithelial markers, BEAS-2B cells expressed 7.1-fold more TJP1 than primary cells from non-smokers (unpaired t-test, $p = 0.0374$).

Overall the primary cells mimicked the pattern of expression seen in the biopsies, however contrary contray to expectations the different groups of primary cells did not differentially express any of the epithelial markers measured. The.T BEAS-2B cell line did not appear to

match the expression levels of the primary cells, and expressed significantly higher levels of TJP1 compared to cells taken from non-smokers.

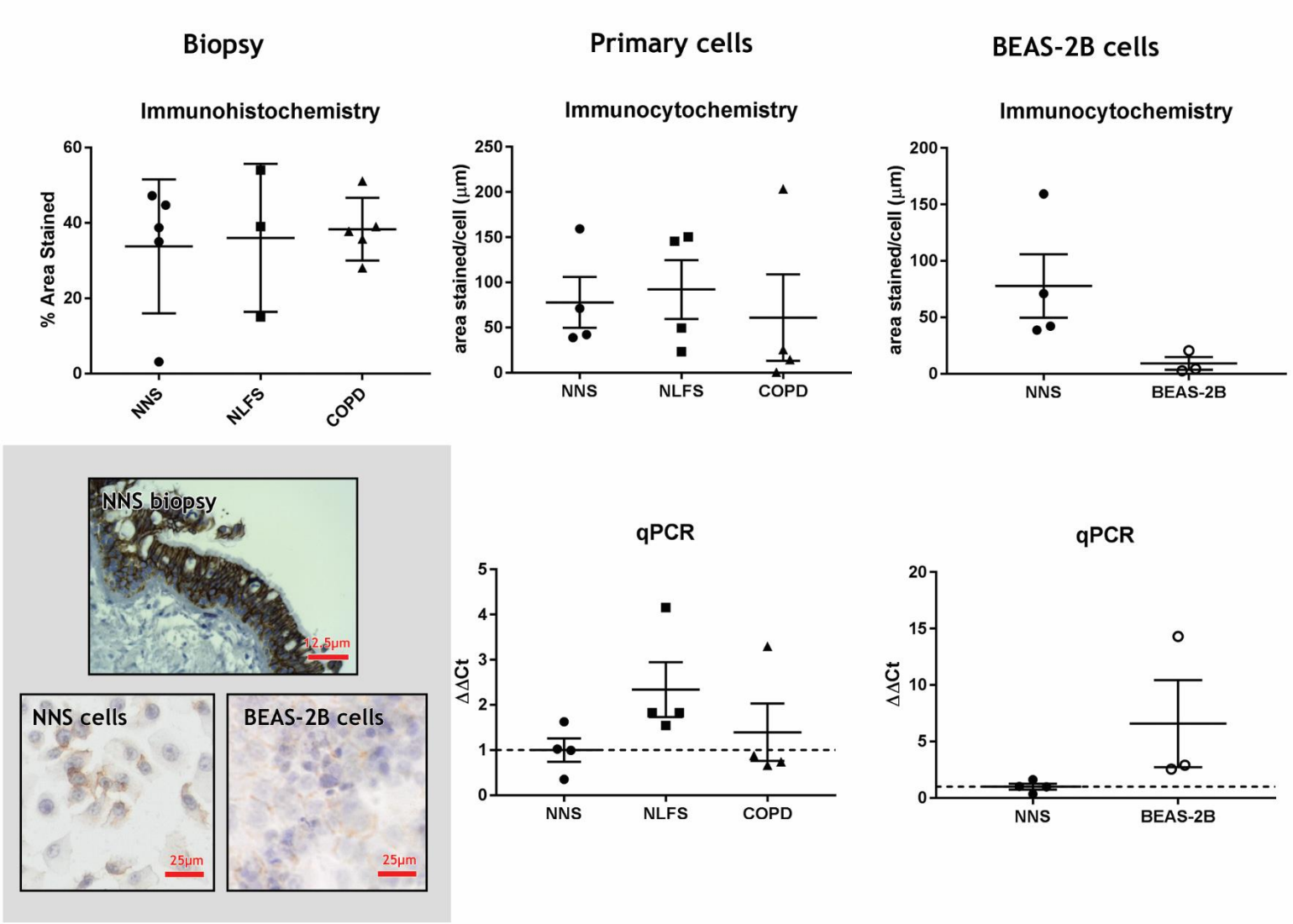

Figure 4.2-1: Expression of E-cadherin in biopsies and primary cell cultures from non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD)people with airflow limitation (COPD) and the BEAS-2B cell line as measured by immunocytochemistry and qPCR. *Data are represented as mean with SEM.* ***Immuno:*** *Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer or experimental sample.* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in non-smokers' cells. The dashed line indicates the expression of the non-smokers' cells. ΔΔCT shows the fold-change in expression, with '1' being no change from baseline or the control.*

In immunocytochemical and immunohistological images, cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin.

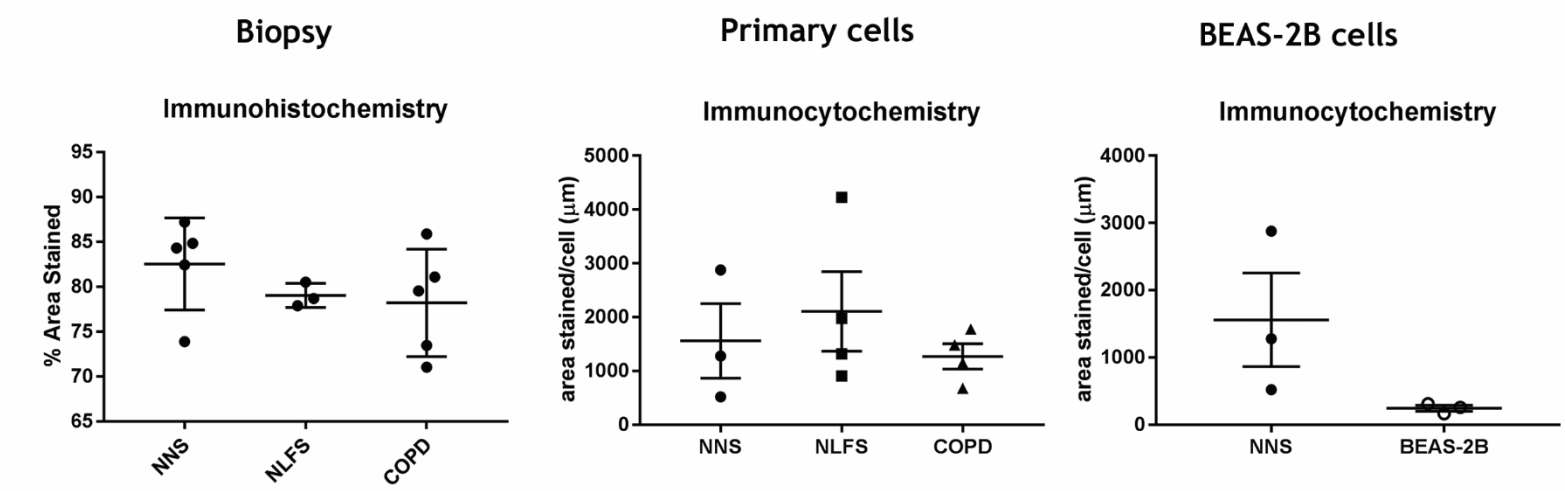

Figure 4.2-2: Expression of cytokeratin in biopsies and primary cell cultures from non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and the BEAS-2B cell line as measured by immunocytochemistry. *Data are represented as mean with SEM. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer.*

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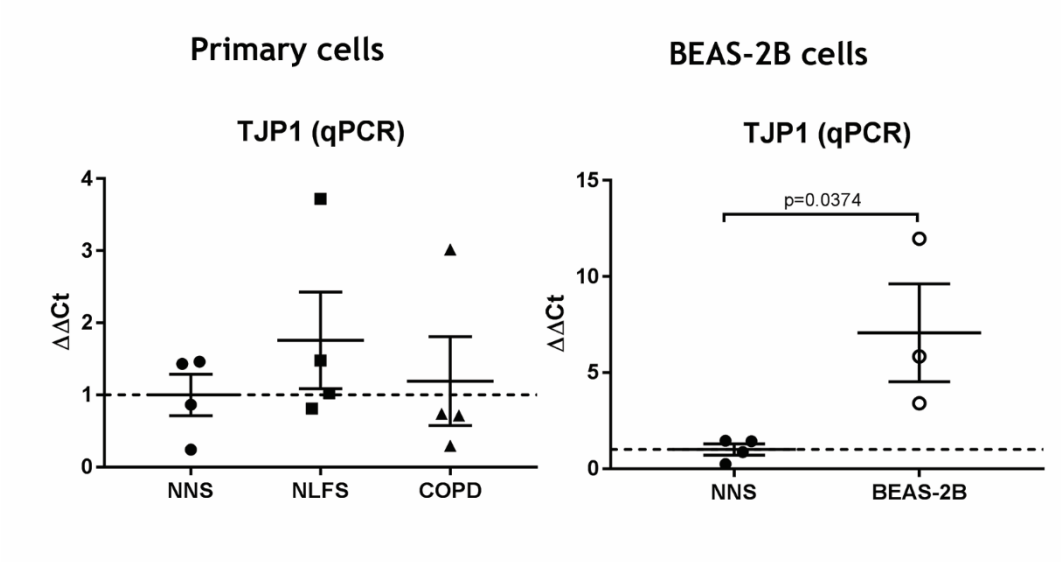

Figure 4.2-3: Expression of tight junction protein-1 (TJP1) in biopsies and primary cell cultures from non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and the BEAS-2B cell line as measured by qPCR. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in non-smokers' cells. The dashed line indicates the expression of the non-smokers' cells.*

4.2.2. Mesenchymal markers

At the protein and mRNA levels the cultured primary cells from non-smokers, smokers with normal lung function and people with airflow limitation agreed with the pattern of equal levels of N-cadherin seen in the biopsies (Figure 4.2-4). However, two of the cultured samples demonstrated levels of N-cadherin protein which appeared elevated when compared to the other samples. BEAS-2B cells, despite a single replicate which an expressed increased level of N-cadherin compared to the other samples, expressed similar levels of N-cadherin as cells from non-smokers, although at the mRNA level they expressed 16.8-fold higher levels of N-cadherin than non-smokers' cells (unpaired t-test $p<0.0001$).

Expression of vimentin in both biopsies and cultured primary cells appeared consistent between non-smokers, smokers with normal lung function and people with airflowairflow limitation, although as seen with N-cadherin a number of cell samples from smokers with normal lung function exhibited high levels of vimentin (Figure 4.2-5). BEAS-2B cells expressed levels of vimentin protein similar to primary cells from non-smokers, however at the mRNA level expressed 6464-fold higher levels of vimentin (one-way ANOVA $p = 0.0421$).

Expression of S100A4 mRNA in the primary cells was consistent across the three groups, as was the expression of the protein in biospies. Once again, BEAS-2B cells expressed 22.9-timestimes higher levels of S100A4 than primary cells from non-smokers (one-way ANOVA $p = 0.0050$). Overall, the primary cells were a reasonably accurate representations of the epithelial layer in the large airways, mimicking patterns of expression well. BEAS-2B cells

matched the primary cells at the protein level, however demonstrated increased mesenchymal features at the molecular level.

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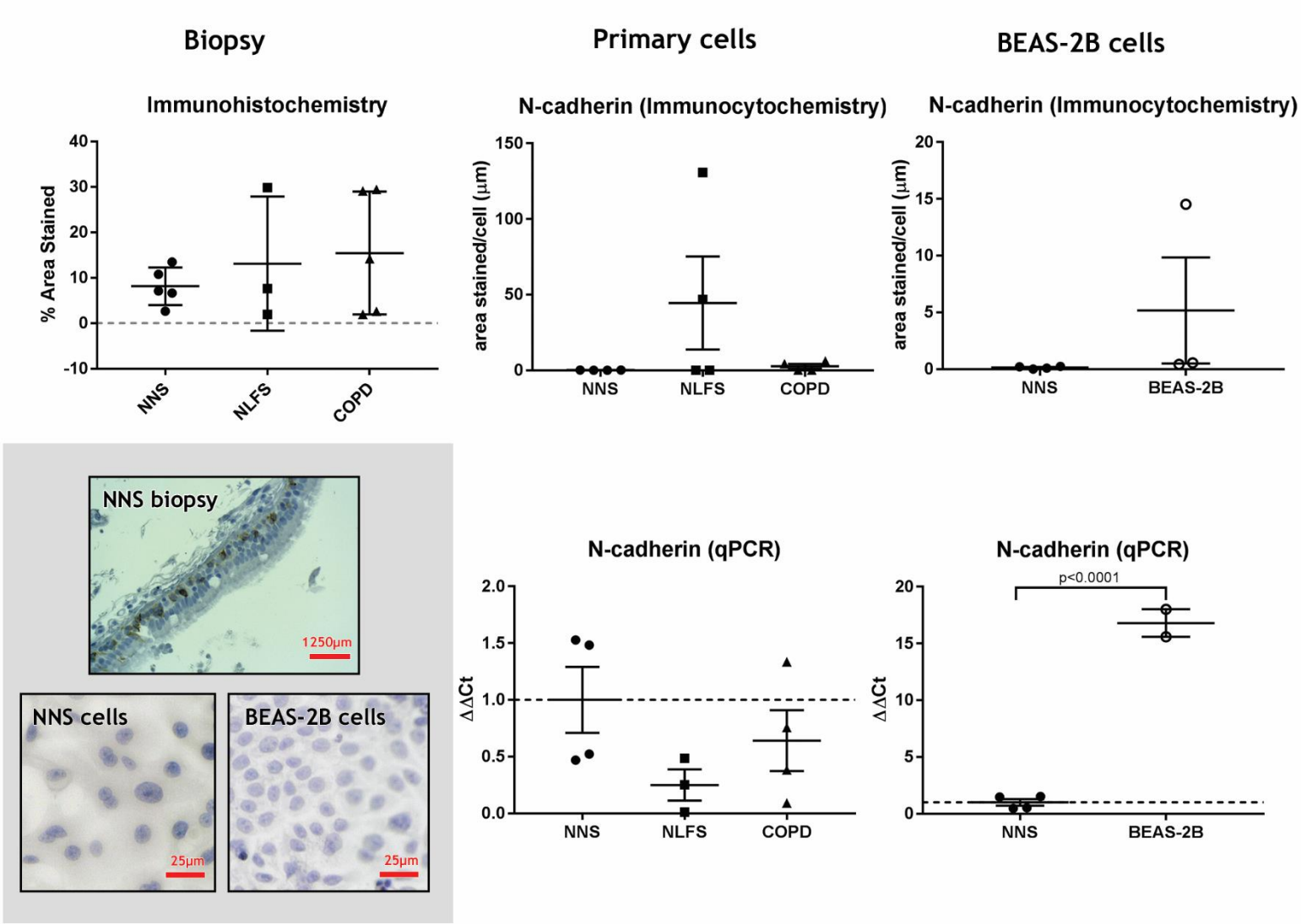

Figure 4.2-4: Expression of N-cadherin in biopsies and primary cell cultures from non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and the BEAS-2B cell line as measured by immunocytochemistry and qPCR. *Data are represented as mean with SEM.* ***Immuno:*** *Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer.* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in non-smokers' cells. The dashed line indicates the expression of the non-smokers' cells.*

In immunocytochemical and immunohistological images, cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin.

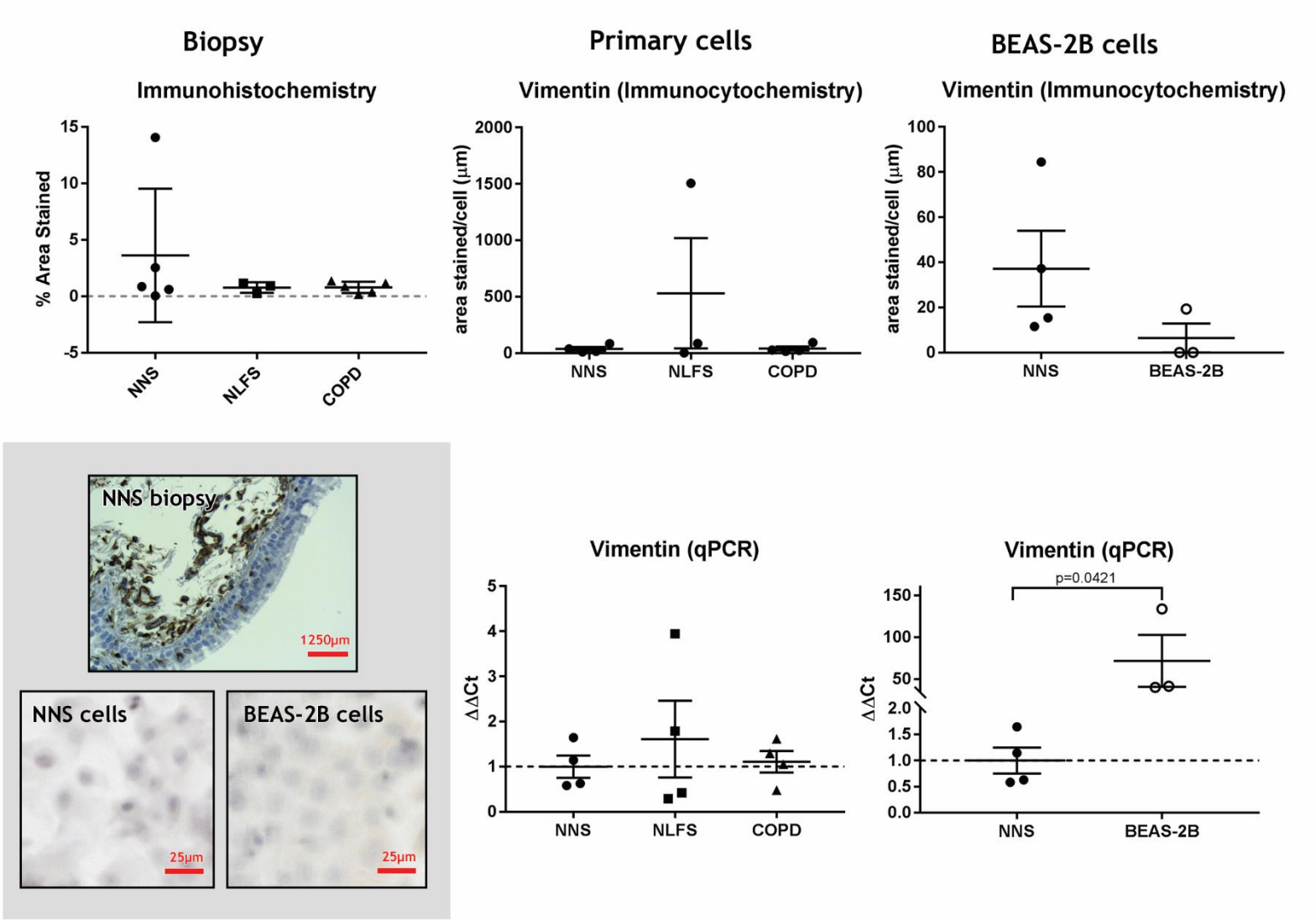

Figure 4.2-5: Expression of vimentin in biopsies and primary cell cultures from non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and the BEAS-2B cell line as measured by immunocytochemistry and qPCR. *Data are represented as mean with SEM.* ***Immuno:*** *Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer.* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in non-smokers' cells. The dashed line indicates the expression of the non-smokers' cells.*

In immunocytochemical and immunohistological images, cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin.

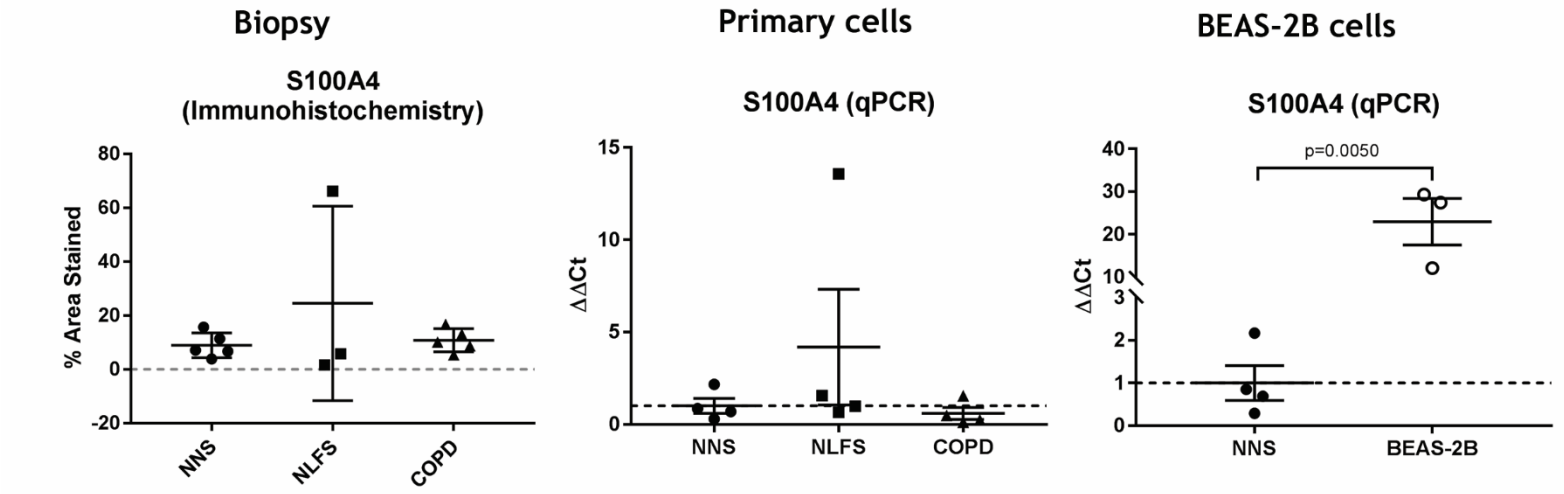

Figure 4.2-6: S100A4 protein and mRNA expression in biopsies taken from non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and the BEAS-2B cell line as measured by immunohistochemistry and qPCR. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in non-smokers' cells. The dashed line indicates the expression of the non-smokers' cells.*

4.2.3. Signalling molecules and extracellular matrix remodelling

Expression of TWISTappeared consistent across all three primary cells groups at both protein and mRNA levels (Figure 4.2-7). BEAS-2B cells expressed levels of TWIST protein equal to those seen in non-smokers' cells, however at the mRNA level there was adecrease in expression, although this did not reach statistical significancesignificance.

In primary cells taken from non-smokers, smokers with normal lung function and people with airflow limitation there nono significant change in Smad6 between non-smokers and the other two groups, despite despitea slight downwards trend in expression (Figure 4.2-8). BEAS-2B cells with minimal variation between samples likewise trended towards decreased expression of Smad6 compared tonon-smokers.

Levels of the extracellular matrix remodelling markers MMP2 and collagen 1-α were similar across all groups of primary cells whether measured at the mRNA level (MMP2 and collagen 1-α) or using the levels of precursor protein secreted into the cell culture supernatant (pro-collagen 1-α) (Figure 4.2-9). However, once again BEAS-2B cells trended towards higher levels of MMP2 and collagen 1-α at the mRNA level than primary cells from non-smokers, although the increase was not translated into increased levels of pro-collagen 1-αalthough.

Overall at the molecular level BEAS-2B cells appeared to vary in expression of both signalling molecules and proteins involved in extracellular matrix formation when compared to primary bronchial epithelial cells from non-smokers. Primary cells expressed these markers consistently across cells taken from non-smokers, smokers with normal lung function and people with airflowairflow limitation.

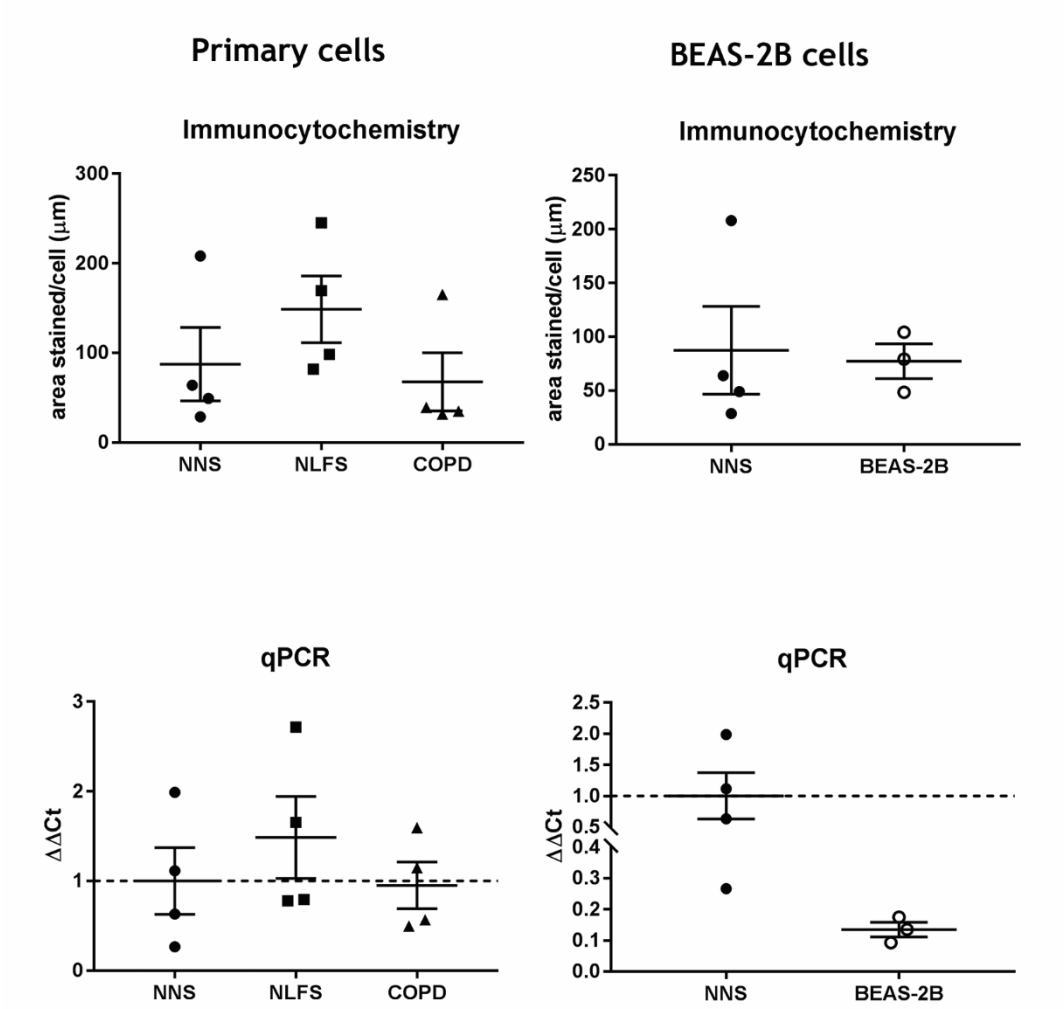

Figure 4.2-7: Expression of TWIST in primary cell cultures from non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and the BEAS-2B cell line as measured by immunocytochemistry and qPCR. *Data are represented as mean with SEM.* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in non-smokers' cells. The dashed line indicates the expression of the non-smokers' cells.*

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CSE *– cigarette smoke extract*

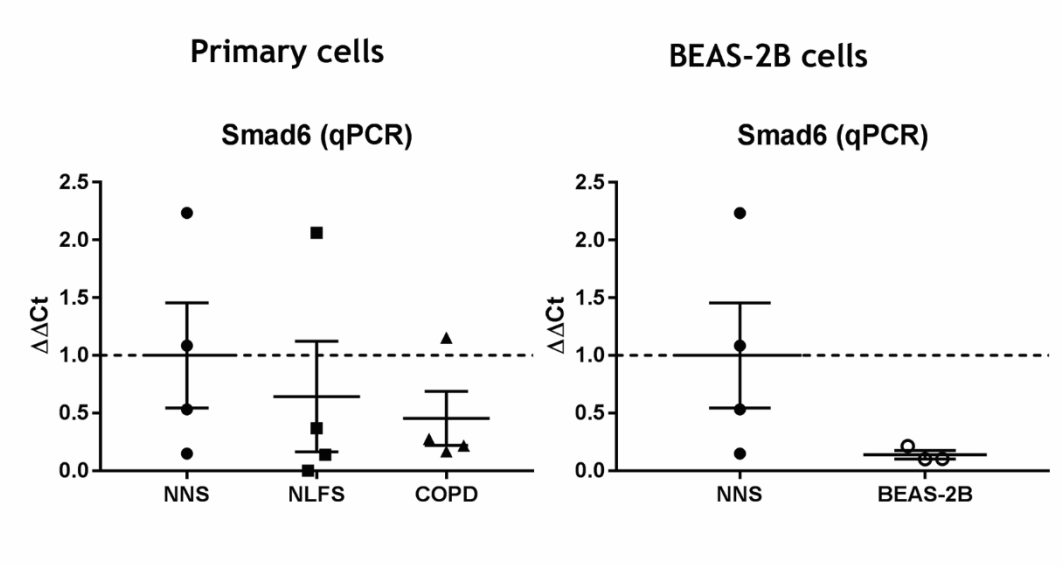

Figure 4.2-8: Smad6 expression primary bronchial epithelial cells taken from non-smokers (NNS), and the BEAS-2B cell line as measured by qPCR. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in non-smokers' cells. The dashed line indicates the expression of the non-smokers' cells.*

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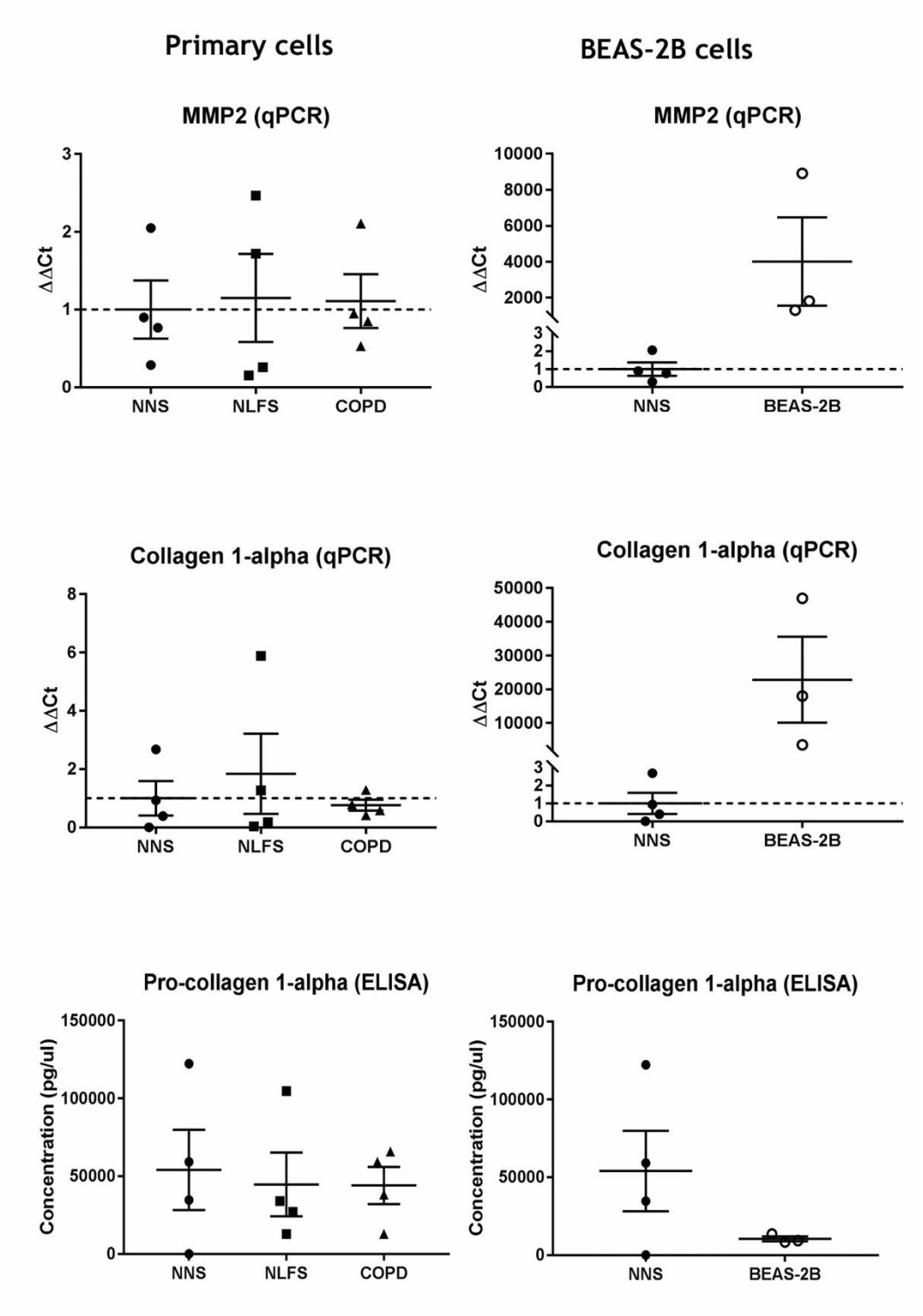

Figure 4.2-9: Expression of MMP2 and collagen 1-α in primary cell cultures from non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and the BEAS-2B cell line as measured by ELISA of cell culture supernatant and qPCR. *Data are represented as mean with SEM.* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in non-smokers' cells. The dashed line indicates the expression of the non-smokers' cells.*

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4.3. Discussion

Primary cells taken from non-smokers, smokers with normal lung function and people with airflow obstruction appeared to accurately model the expression of epithelial-mesenchymal transition (EMT) markers, at least with regards to the patterns of expression seen between the three groups in biopsies. Levels of epithelial, mesenchymal and extracellular matrix markers remained consistent across all groups, indicating that primary bronchial epithelial cells can be used as an *in vitro* model of *in vivo* expression in the bronchial epithelium.

Although this study was underpowered to detect statistically significant changes in the expression of EMT markers, the results suggested that the BEAS-2B cell line is likely to be an inaccurate representation of healthy primary human bronchial epithelial cells, especially when observing molecular changes at the mRNA level. The BEAS-2B cell line appeared to be less epithelial than primary cells from non-smokers, in addition to expressing increased levels of mesenchymal markers and extra-cellular matrix remodelling mRNA, although these changes did not often correspond to differences in protein expression. The increase in mesenchymal markers vimentin and N-cadherin were statistically significant, and the BEAS-2B cells also trended towards increased expression of MMP2 and procollagen 1-α compared to primary cells from non-smokers.. This was likely due to a combination of the small sample size and the fact that the cells were not synchronised in their cell cycle stage, which is likely responsible for the large inter-sample variation seen in the BEASBEAs-2B cells. When attempting to confirm these changes, it is recommended that the cells be synchronised by serum starvation or application of a treatment such as a DNA synthesis inhibitor or a mitotic inhibitor such as nocodazole, depending on the phase of the cell cycle desired. Serum starvation, which synchronises cells in the G0/G1 phase, would be recommended for these

particular studies. Since BEGM media is naturally serum free, in this case 'serum starvation' is technically not the correct term – in order to starve the cells, the basal media without any growth factors (BEBM) would be used instead of the fully supplemented BEGM. Despite these limitations, it was clear thatthe the BEAS-2B cells differeddiffer markedly in their EMT-related profile compared to primary cells from non-smokers, at least at the mRNA level.

This raised the question that, if these differences between immortalised and primary cell lines were only measurable at the mRNA level then perhaps there were differences between the primary cells and *in vivo* tissue which were likewise only apparent at the molecular level. Indeed, perhaps the BEAS-2B cell line more accurately represents the mRNA expression profile of bronchial epithelium *in vivo* than the primary cells? Attempts were made during the study to apply RNAScope to biopsies matched to those taken for protein analysis (section 2.7.3) in order to quantify mRNA expression in the tissue itself. However, despite several attempts at optimisation the RNAScope platform failed to stain the sections for the housekeeping gene *Peptidylprolyl Isomerase B* (PPIB). This was considered likely due to the fact that the sections were collected and preserved prior to the release of the RNAScope recommended guidelines for treatment of samples during collection. DrBased on out lab's previous experience with in-situ hybridisation of tissue samples, the tissue was treated as described in the Methods and Materials, however the fixation times were far longer than recommended for the RNAScope kit. The original samples remain in storage and in future a technique such as formalin-fixed paraffin-embedded (FFPE) RNA extraction and analysis could be performed to examine differences in molecular level expression of markers,

although the epithelial layer would need to be isolated prior to extraction, possibly by laser-dissection, in order to get a useful answer.

Further study to confirm if the differences between BEAS-2B cells and primary cells seen in this study are real is required, however it would appear likely that even if the BEAS-2B cell line differs from healthy primary bronchial epithelial cells at the molecular level, if the changes do not extend to protein expression then the differences are unlikelyunlikely to be of functional significance. In this case, it would be prudent to preferentially examine protein-level changes when utilising the BEAS-2B cell line as a model for healthy primary bronchial epithelial cells, as the molecular-level characteristics appeared to not be an accurate representation of expression.

While this studywas primarily interested in the viability of primary and immortalised cell culture systems as models for *in vivo* bronchial epithelium, the results obtained from the biopsy tissue raised some mild concerns. Previously, the literature has demonstrated that EMT is active in COPD [31-33, 39, 40, 207], with increased expression of mesenchymal markers and decreased levels of epithelial markers. The results seen here do not match these data, however this is likely due to the fact that the volunteers recruited in this study did not have full, clinical COPD. While they did exhibit airflow limitation, it is possible that EMT may not be fully active at such an early stage of disease development. This means that, although BEAS-2B cells appeared to be unsuitable models of healthy airway epithelium, it is possible that they may still be useful for studying COPD-related disease, as they may share characterisitics with cells taken from established disease. Further work using cells taken from people with established, clinical COPD is recommended.

Additionally, the biopsies in the current study were not randomly selected – instead, biopsies which were paired to cell cultures which grew and survived well and produced a high number of cells for use were used, in order to produce paired data. Although this sort of selection bias is difficult to avoid in cell culture, it may have inadvertently caused biopsies from individuals with 'healthier' epithelium to have been selected for among the cohort with airflow limitation. This may have obscured any EMT signal produced by the airflow limitation, as the cells which grew well would, theoretically, approximate 'normal' cells in terms of their phenotype, and thus the biopsies' epithelium would appear closer to healthy than diseased.

Secondly, in the literature [31, 32, 40, 255] only cells within 10μm of the reticular basement membrane were counted (personal correspondence, Sukwinder Sohal 02/Feb/2017), and were referred to as 'basal cells'. While in the case of stains such as E-cadherin or cytokeratin, which appeared to stain the entire epithelial layer with reasonable uniformity, this distinction is likely unimportant, N-cadherin and S100A4 demonstrated more discrete, point staining and, at least in the case of N-cadherin, appeared to localise in proximity to the basement membrane. Thus, it is possible that the difference in counting methodology contributed to the differences between the data in this study and the literature, although this is effect may be overshadowed by the fact that the cells used in this study did notdi dnot come from people with clinical COPD.

In this regard, it is also worthwhile to note that the cell culture data obtained in this study did not agree with the results previously seen in the literature [33], which indicate a maintenance of an EMT phenotype in culture. However, as previously mentioned, the cells in this study were obtained from volunteers with pre-COPD, rather than established disease, which potentially explains the discrepancy. As the cells in this study agreed with their paired

biopsies with regards to the lack of presence of an EMT phenotype, this issue does not have a great impact on the results of this study.

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

4.4. Chapter conclusions

Primary cells taken from non-smokers, smokers with normal lung function and people with airflow limitation appeared to exhibit a phenotype in culture which matched that seen in the epithelium of biopsies from similar subjects, despite undergoing two passages and cryopreservation. This suggests that they are, as is generally accepted, a reliable model of human bronchial epithelial cells *in vivo*. However, the BEAS-2B cell line, which is commonly used as a stand-in for healthy primary bronchial epithelial cells, appeared to exhibit an altered, mesenchymal-like, phenotype at the molecular level when compared to primary cells taken from non-smokers. This altered phenotype did not translate into changes at the protein level, suggesting that BEAS-2B cells may still be applicable for functional studies where non-smoker primary cells are unavailable, however analysis should focus on protein level changes rather than relying on mRNA wherever possible. While this study suggests that BEAS-2B cells may be an unreliable model of healthy bronchial epithelium, if they react in the same way to stimulus as the pHBECs then they may still prove to be a useful model, despite differences in their baseline phenotype.

5. Artificial induction of EMT in healthy bronchial epithelial cells and its relevance to EMT in COPD

5.1. Introduction

From the literature, EMT is known to occur in COPD airways [31-34], and epithelial cells cultured from people with COPD exhibit abnormal growth and differentiation, in addition to an EMT phenotype [26, 27, 33, 103, 235, 256]. This makes bronchial epithelial cells taken from COPD airways, which exhibit a changed phenotype that endures in culture a valuable resource for identifying pathways and mechanisms. However, they are challenging to obtain and difficult to work with. SinceS EMT can be induced in both primary and immortalised bronchial epithelial cells lines, is it possible to replicate the EMT phenotype seen in COPD in these cells, which are easier to obtain and simple to work with?

5.1.1. The CSE-TGF-β-EMT pathway

Exposure to cigarette smoke has been shown to induce EMT in both pHBECs and immortalised bronchial epithelial cells [33], as well as in alveoli-derived epithelial cells [203]. There have been a number of pathways implicated in cigarette smoke extract (CSE) induced EMT, including the Rac1 pathway [203], ERK1/2 phosphorylation, modulation of cyclic monophosphate and TGF-β signalling via Smad3 [33].

Although TGF-β may not be the only factor modulating EMT in response to CSE, it is well known to be a reliable inducer of EMT on its own [33, 165, 257-260], as well as being involved in a number of diseases which involve epithelial-mesenchymal transition (EMT), including hepatic fibrosis [65, 80], kidney fibrosis [58, 79, 81] and non-COPD-related pulmonary fibrosis [58, 82]. Furthermore, TGF-β has been shown to have increased receptor expression in the acinar mucus glands in COPD airways [261], and has also been shown to be increased in the airway epithelium, where it is speculated to play a role in the recruitment of macrophages [85]. Cigarette smoke, potentially via TGF-β, is thought to drive EMT in COPD, likely through either the Smad signalling pathway [88, 89], or the Smad-independent MAPK pathway [90]. As both TGF-β and CSE are known to induce EMT in bronchial epithelial cells in culture, it is interesting to know whether CSE induced EMT is the same as EMT induced by TGF-β alone. This study aimeded to examine the similarities and differences between CSE induced EMT and TGF-β induced EMT, with an eye to determining whether TGF-β signalling via the Smad pathway is the key factor in the CSE-TGF-β-EMT pathway postulated to occur in COPD and chronic airflow limitation.

5.1.2. Chapter aimsa

The aims of this were as follows:

- To determine if primary bronchial epithelial cells taken from non-smokers react to transforming growth factor-β1 in the same manner as the commercially available immortalised BEAS-2B cell line with regards to epithelial-mesenchymal transition (EMT) markers, two signalling pathways and expression of two extracellular remodelling factors.
- To determine if primary bronchial epithelial cells taken from non-smokers react to cigarette smoke extract in the same manner as the commercially available immortalised BEAS-2B cell line with regards to EMT markers, two signalling pathways and expression of two extracellular remodelling factors.

Hypothesis: BEAS-2B cells and primary bronchial epithelial cells taken from non-smokers will exhibit changes of similar magnitude in EMT-related markers in response to transforming growth factor-β1 or cigarette smoke extract.

5.2. Results

5.2.1. CSE

5.2.1.1. Epithelial markers

E-cadherin demonstrated no change in expression following exposure to cigarette smoke extract (CSE) in either primary or BEAS-2B cells (Figure 5.2-1). SimilarlySimilarly, cytokeratin (Figure 5.2-2) and tight junction protein-1 (Figure 5.2-3) did not change in response to CSE. Overall, CSE appeared to have no effect on epithelial marker expression.

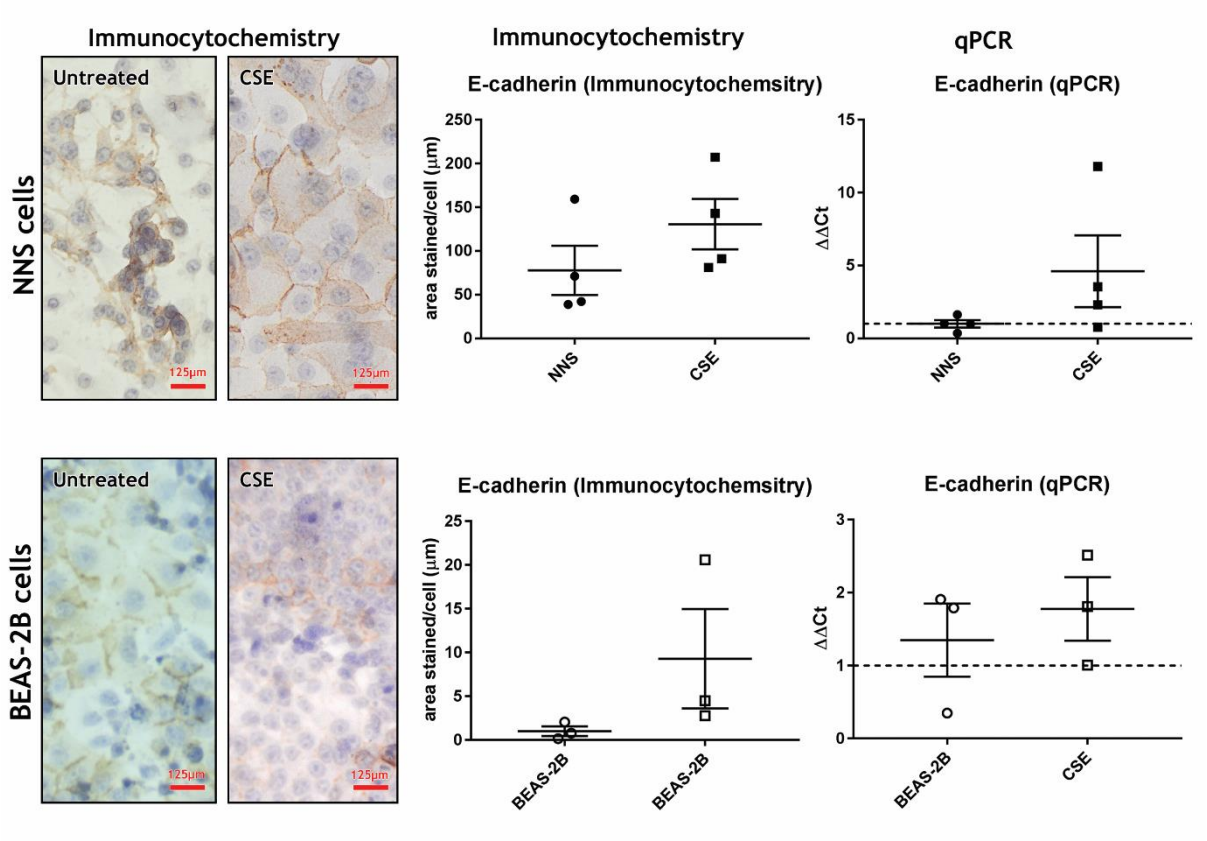

Figure 5.2-1: Expression of E-cadherin in primary bronchial epithelial cells from non-smokers (NNS) and the immortalised BEAS-2B cell line following exposure to cigarette smoke extract (CSE) for 4 hours as measured by immunocytochemistry and qPCR. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are NNS pHBECs, open shapes are BEAS-2B cells.*

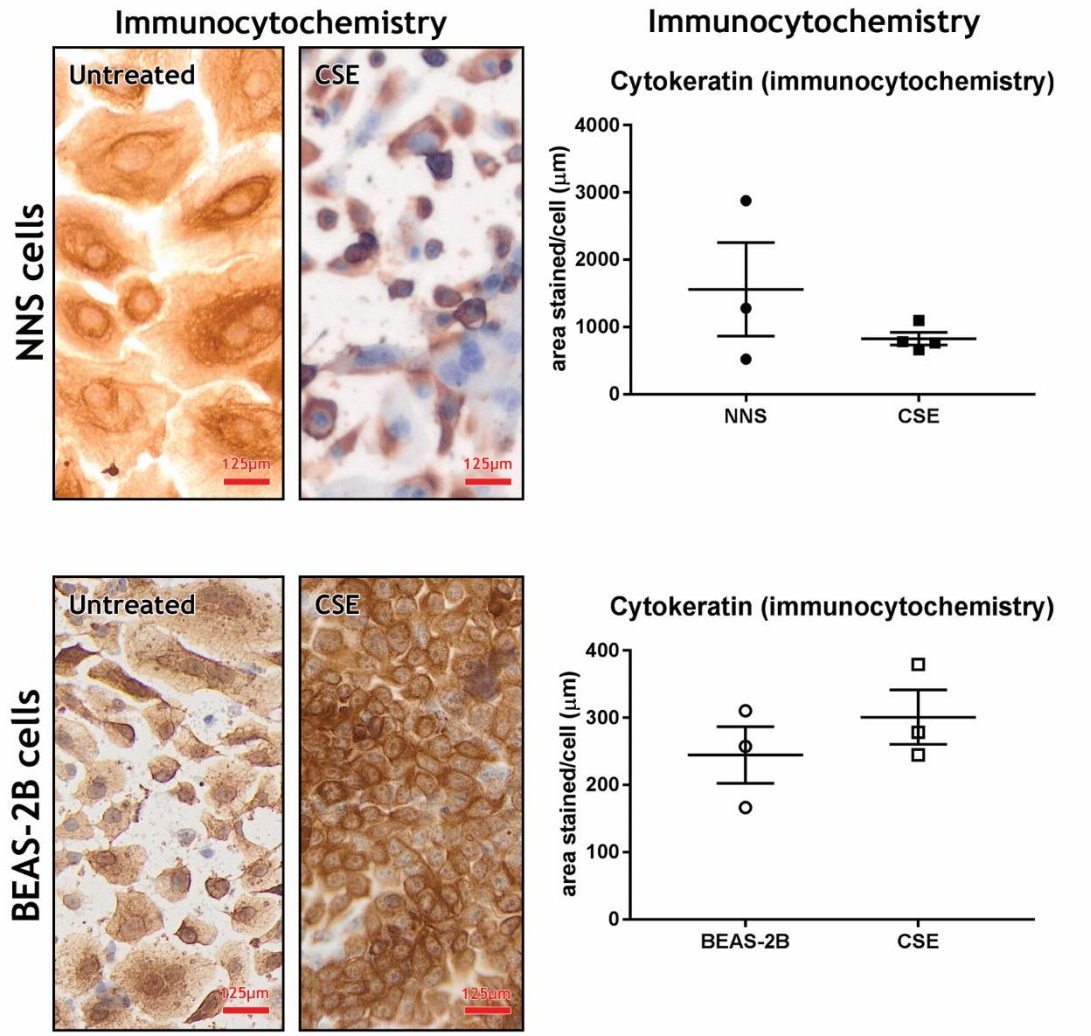

Figure 5.2-2: Expression of cytokeratin in primary bronchial epithelial cells from non-smokers (NNS) and the immortalised BEAS-2B cell line following exposure to cigarette smoke extract (CSE) for 4 hours as measured by immunocytochemistry. *Data are represented as mean with SEM. In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).*

Key: *Coloured shapesFilled shapes are NNS pHBECs, open shapes are BEAS-2B cells.*

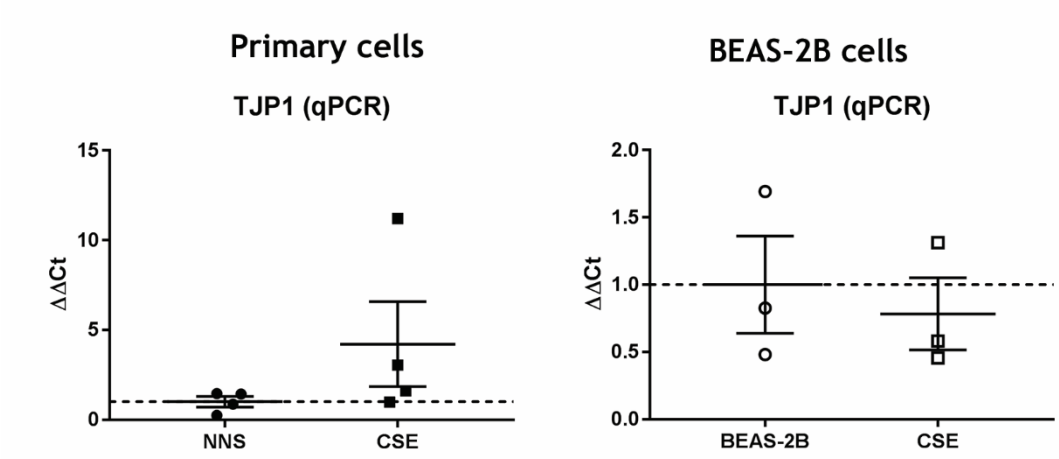

Figure 5.2-3: Expression of tight junction protein-1 (TJP1) in primary bronchial epithelial cells from non-smokers (NNS) and the immortalised BEAS-2B cell line following exposure to cigarette smoke extract (CSE) for 4 hours as measured by qPCR. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are NNS pHBECs, open shapes are BEAS-2B cells.*

5.2.1.2. Mesenchymal markers

Exposure to cigarette smoke extract (CSE) increased N-cadherin protein expression by 405.3 times in primary cells (Welch's t-test p = 0.0363), however had no effect on protein expression in BEAS-2B cells (Figure 5.2-4). At the mRNA level, CSE had no effect on N-cadherin expression in primary cells, although BEAS-2B cells exhibited an 87.3-fold decrease in expression (Welch's t-test p = 0.0459) following exposure.

Protein and mRNA levels of vimentin expression were unchanged in both cell types following CSE exposure (Figure 5.2-5). Levels of S100A4 mRNA were unchanged by CSE (Figure 5.2-6). Overall, CSE increased expression of mesenchymal markers in primary cells while suppressing expression in BEAS-2B cells, although not all mesenchymal markers were affected.

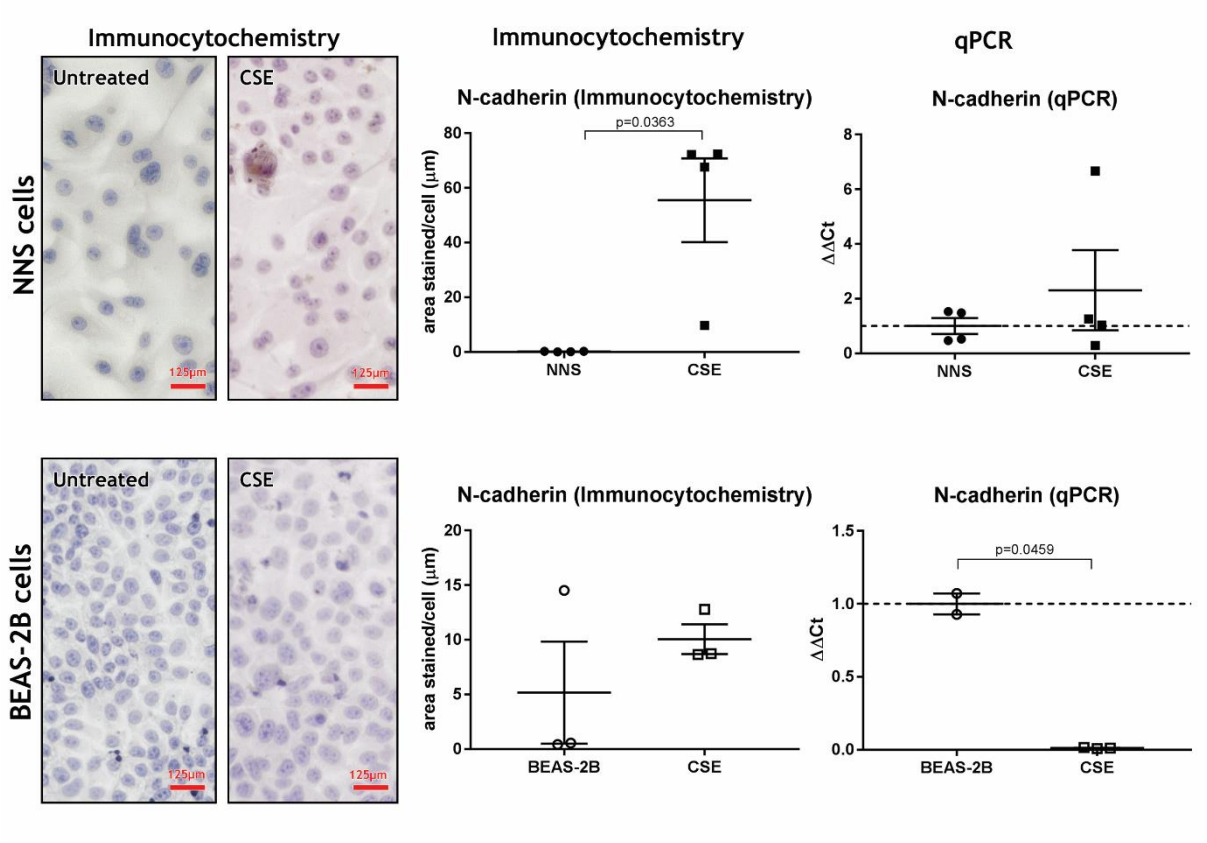

Figure 5.2-4: Expression of N-cadherin in primary bronchial epithelial cells from non-smokers (NNS) and the immortalised BEAS-2B cell line following exposure to cigarette smoke extract (CSE) for 4 hours as measured by immunocytochemistry and qPCR. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

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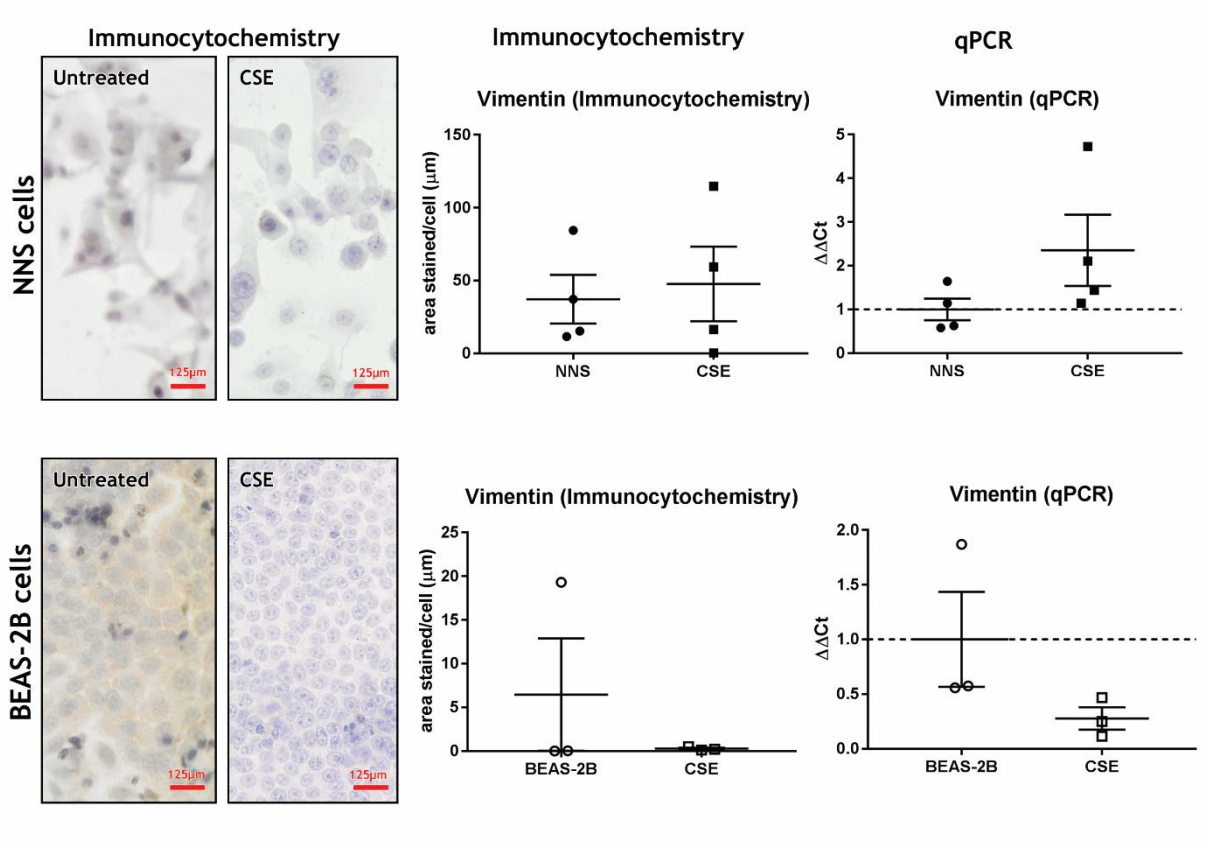

Figure 5.2-5: Expression of vimentin in primary bronchial epithelial cells from non-smokers (NNS) and the immortalised BEAS-2B cell line following exposure to cigarette smoke extract (CSE) for 4 hours as measured by immunocytochemistry and qPCR. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

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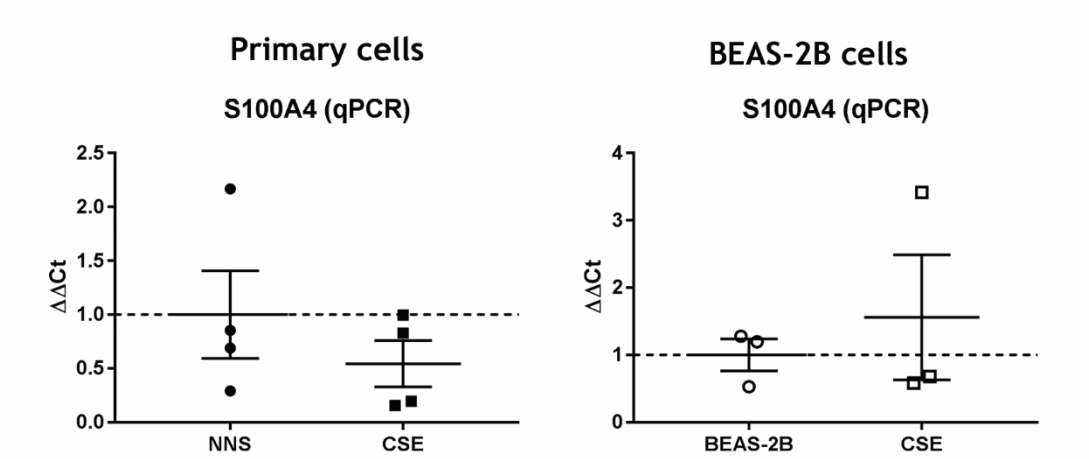

Figure 5.2-6: Expression of S100A4 in primary bronchial epithelial cells from non-smokers (NNS) and the immortalised BEAS-2B cell line following exposure to cigarette smoke extract (CSE) for 4 hours as measured by qPCR. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are NNS pHBECs, open shapes are BEAS-2B cells.*

5.2.1.3. Signalling molecules

TWIST expression at the protein level was unaffected by exposure to cigarette smoke extract (CSE) in both primary and BEAS-2B cells (Figure 5.2-7). Smad6 expression was unaffected by CSE in primary cells (Figure 5.2-8). Overall, the TWIST and Smad pathways were unaffected by CSE., despite suggestions of mild non-significant changes in expression at the mRNA level.

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

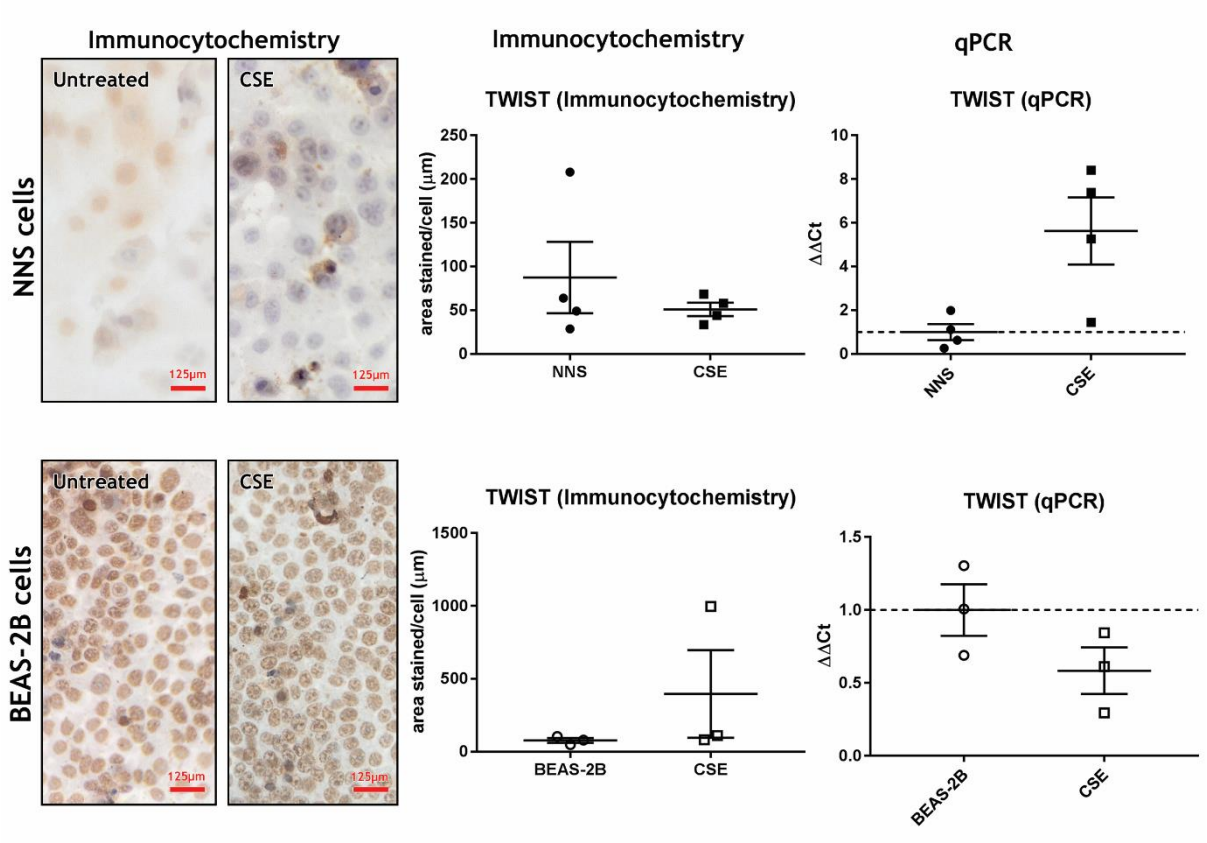

Figure 5.2-7: Expression of TWIST in primary bronchial epithelial cells from non-smokers (NNS) and the immortalised BEAS-2B cell line following exposure to cigarette smoke extract (CSE) for 4 hours as measured by immunocytochemistry and qPCR. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

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TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

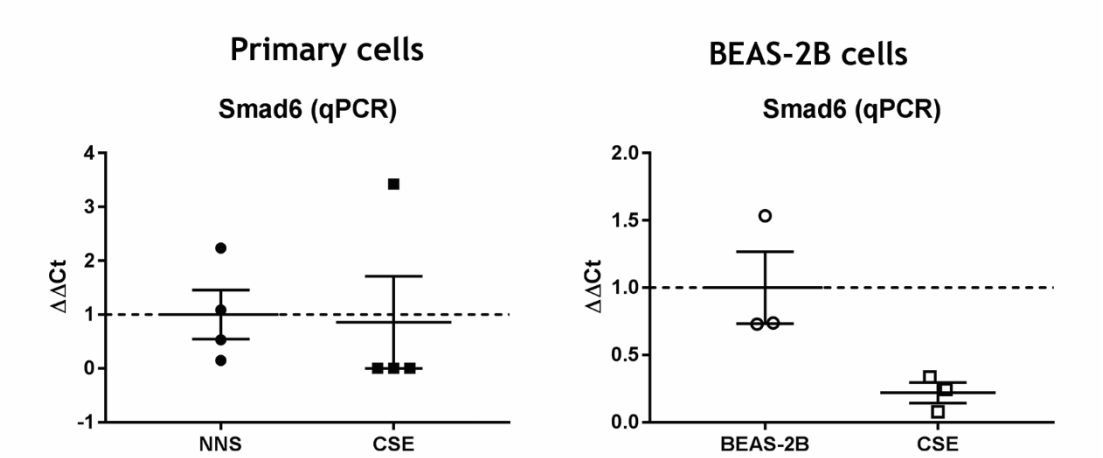

Figure 5.2-8: Expression of Smad6 in primary bronchial epithelial cells from non-smokers (NNS) and the immortalised BEAS-2B cell line following exposure to cigarette smoke extract (CSE) for 4 hours as measured by qPCR. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are NNS pHBECs, open shapes are BEAS-2B cells.*

5.2.1.4. Extracellular remodelling

MMP2 expression in the primary cells remained broadly unchanged by cigarette smoke extract (CSE) exposure, although a single sample did demonstrate a large increase in response to the treatment (Figure 5.2-9). In contrast, expression of MMP2 in the BEAS-2B decreased by 856.2-fold, although the changeit did not reach statistical sginificance.

Both primary and BEAS-2B cells demonstrated a decrease in expression of collagen 1-α mRNA following CSE exposure, and while this change did not reach statistical significance there was a statistically significant decrease in the level of pro-collagen 1-α protein in the cell culture supernatant, it being too low to be detected in both primary and BEAS-2B cells following exposure ([primary] Welch's t-test $p = 0.0328$; [BEAS-2B] Wlech's t-test $p<0.001$) (Figure 5.2-9). Expression of extracellular matrix remodelling proteins appeared to be downregulated in both BEAS-2B and primary cells in response to CSE.

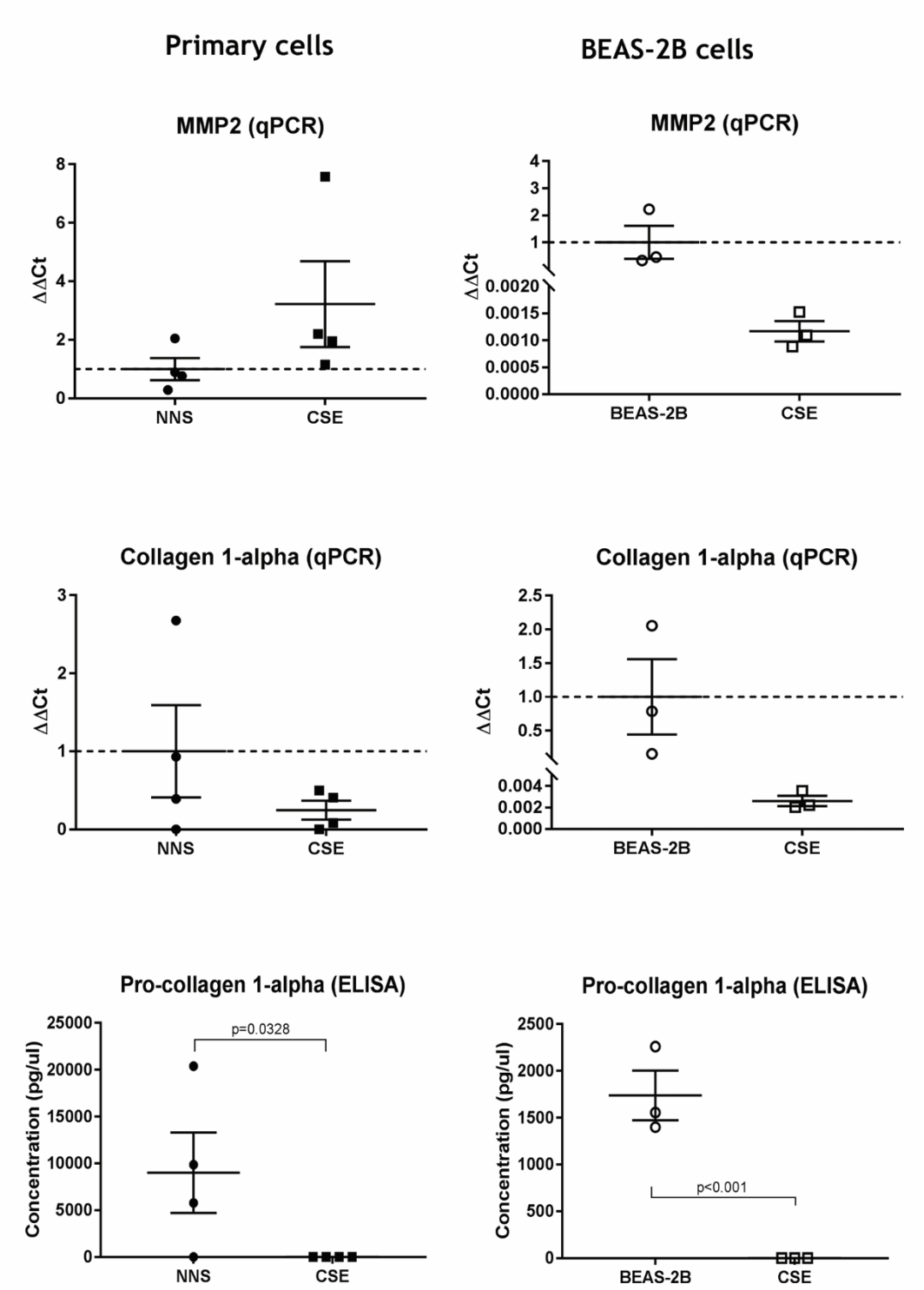

Figure 5.2-9: Expression of MMP2 and collagen 1-α in primary bronchial epithelial cells from non-smokers (NNS) and the immortalised BEAS-2B cell line following exposure to cigarette smoke extract (CSE) for 4 hours as measured by ELISA of cell culture supernatant and qPCR. *Data are represented as mean with SEM.* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are NNS pHBECs, open shapes are BEAS-2B cells.*

Useful abbreviations

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COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

5.2.2. TGF-β

5.2.2.1. Epithelial markers

Exposure to transforming growth factor-β1 (TGF-β) had no effect on E-cadherin expression in either primary or BEAS-2B cells (Figure 5.2-10). Likewise, both cytokeratin (Figure 5.2-11) and tight junction protein-1 expression (Figure 5.2-12) werewere unaffected in both cell types. Overall, TGF-β had no effect on epithelial marker expression.

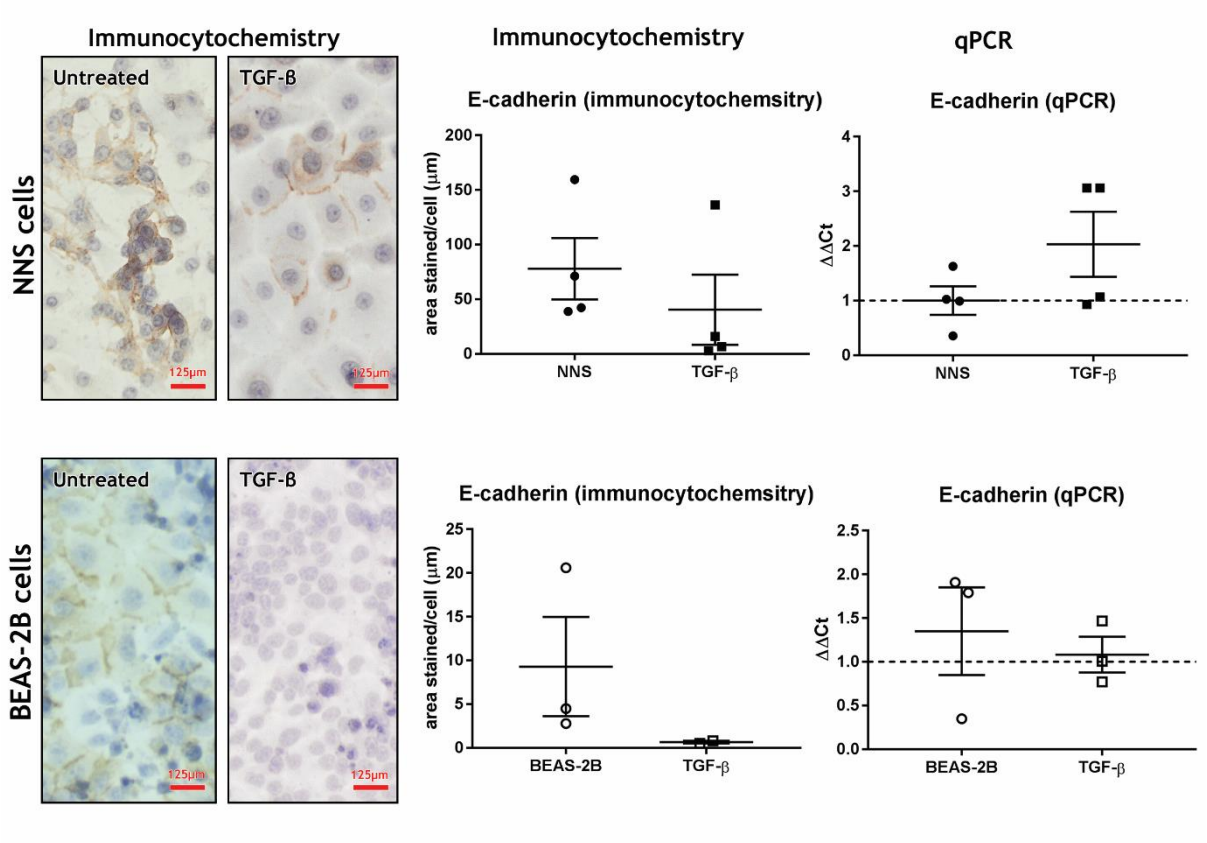

Figure 5.2-10: Expression of E-cadherin in primary bronchial epithelial cells from non-smokers (NNS) and the immortalised BEAS-2B cell line following exposure to transforming growth factor-β1 (TGF-β) for 72 hours as measured by immunocytochemistry and qPCR. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are NNS pHBECs, open shapes are BEAS-2B cells.*

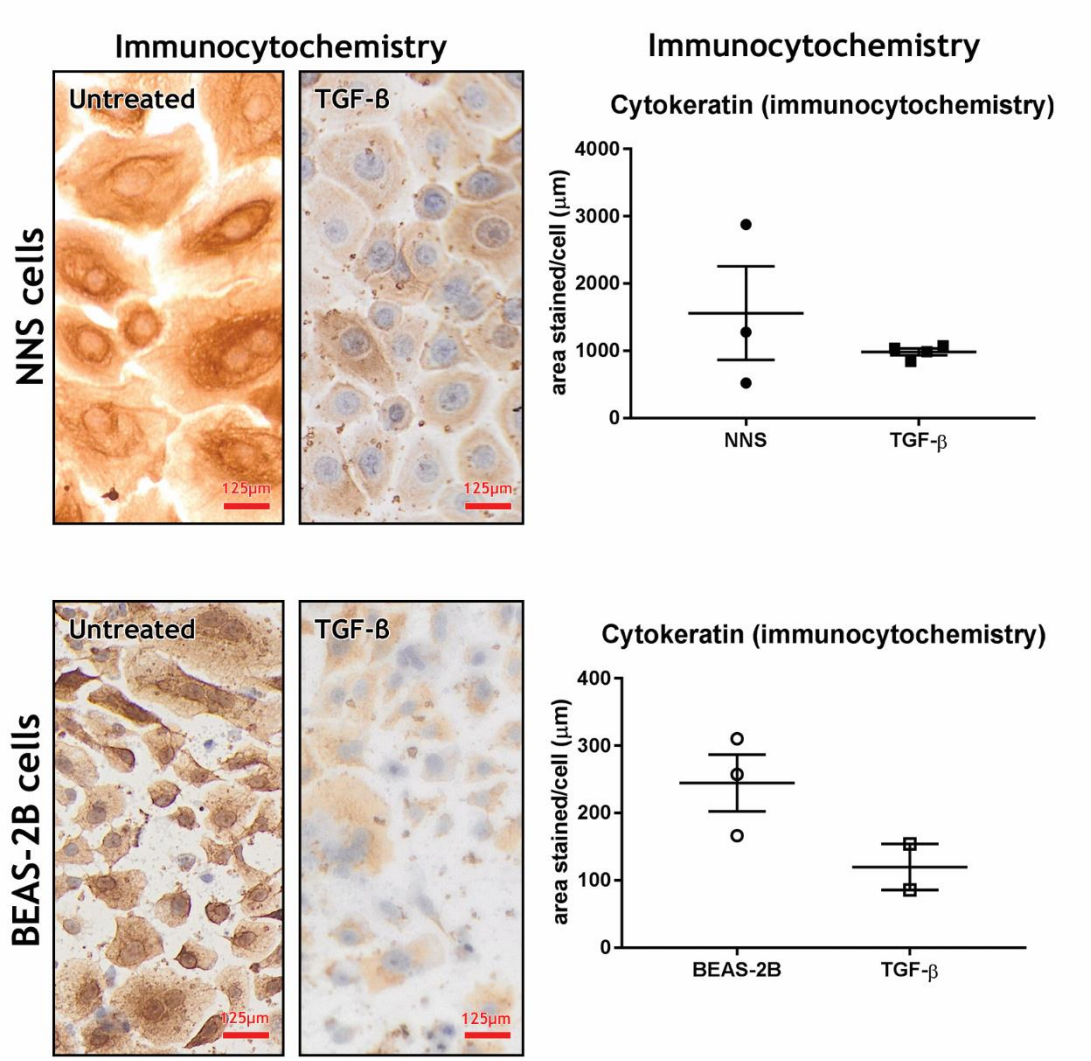

Figure 5.2-11: Expression of cytokeratin in primary bronchial epithelial cells from non-smokers (NNS) and the immortalised BEAS-2B cell line following exposure to transforming growth factor-β1 (TGF-β) for 72 hours as measured by immunocytochemistry. *Data are represented as mean with SEM. In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).*

Key: *Coloured shapesFilled shapes are NNS pHBECs, open shapes are BEAS-2B cells.*

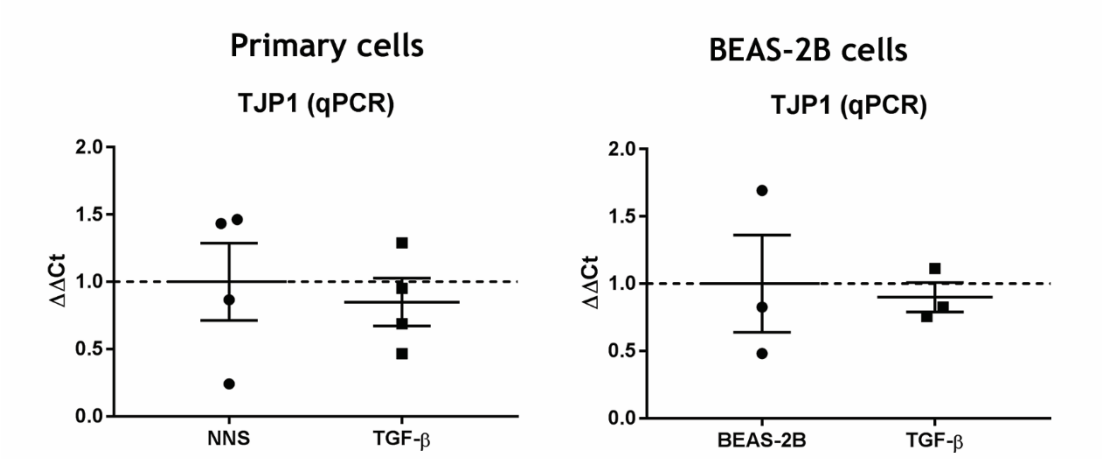

Figure 5.2-12: Expression of tight junction protein-1 (TJP1) in primary bronchial epithelial cells from non-smokers (NNS) and the immortalised BEAS-2B cell line following exposure to transforming growth factor-β1 (TGF-β) for 72 hours as measured by qPCR. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are NNS pHBECs, open shapes are BEAS-2B cells.*

5.2.2.2. Mesenchymal markers

Primary cells treated with TGF-β, despite showing a 19.1-fold decrease (one-way ANOVA p = 0.0461) in N-cadherin at the mRNA level showedshowed no change in N-cadherin expression at the protein level in response to TGF-β (Figure 5.2-13). Similarly, although BEAS-2B cells showed a 63.5-fold decrease (one-way ANOVA p<0.0001) in mRNA expression of N-cadherin, the protein level remained unchanged following TGF-β exposure.

A downwards trend in vimentin mRNA expression was observed in both primary and BEAS2-2B cells, however this did not reach statistical significance and therethere was no change in protein level expression (Figure 5.2-14). S100A4 remained unaffected by TGF-β in both primary and BEASS-2B cells (Figure 5.2-15).

Overall, while TGF-β appeared to have a suppressive effect on mRNA levels of mesenchymal markers in both primary and BEAS-2B cells, this did not result in changed protein level expression.

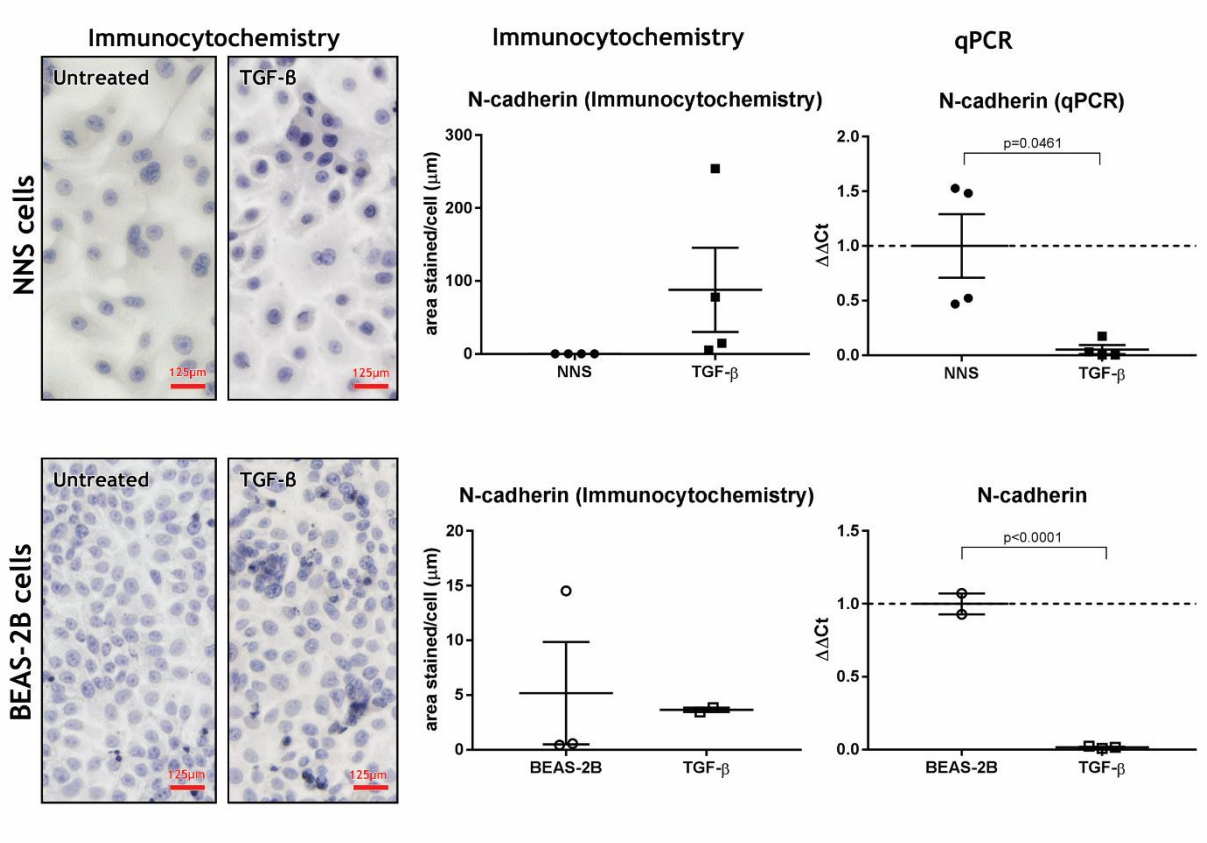

Figure 5.2-13: Expression of N-cadherin in primary bronchial epithelial cells from non-smokers (NNS) and the immortalised BEAS-2B cell line following exposure to transforming growth factor-β1 (TGF-β) for 72 hours as measured by immunocytochemistry and qPCR. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

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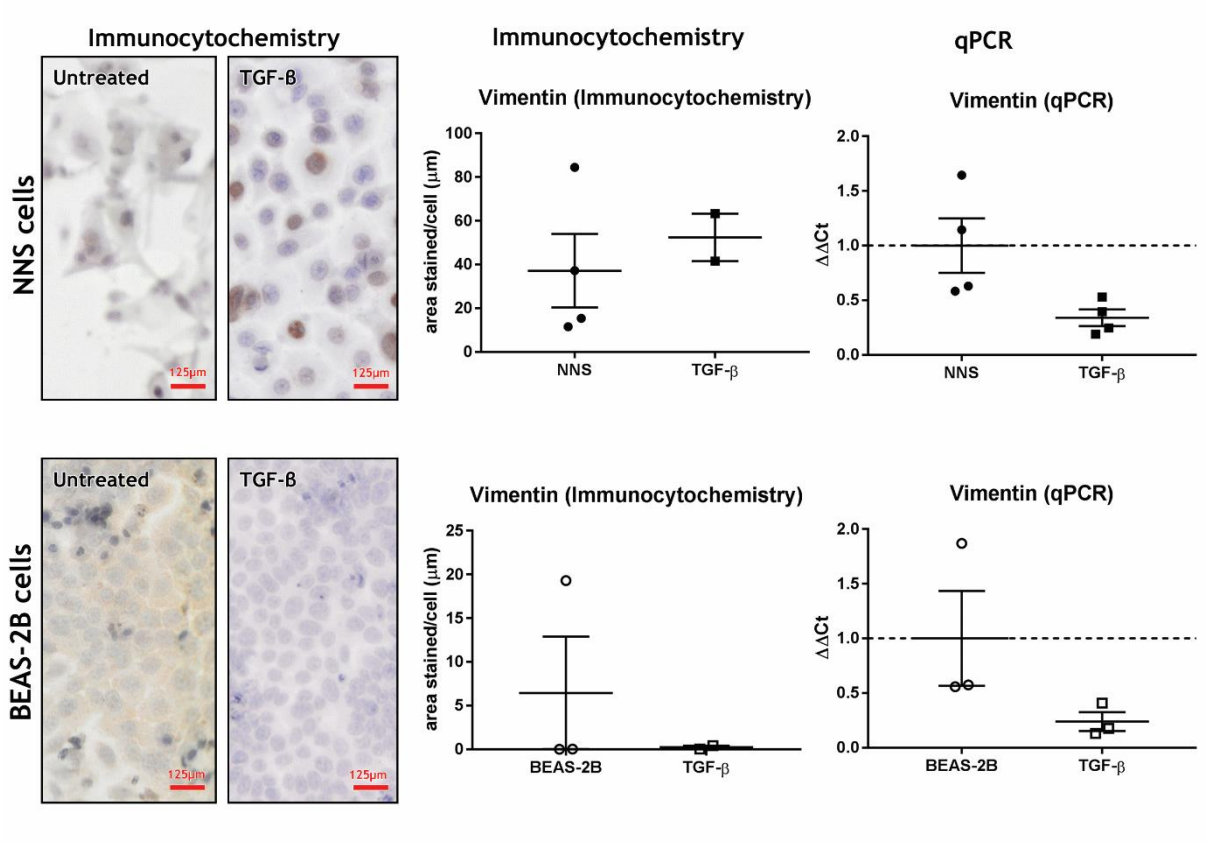

Figure 5.2-14: Expression of vimentin in primary bronchial epithelial cells from non-smokers (NNS) and the immortalised BEAS-2B cell line following exposure to transforming growth factor-β1 (TGF-β) for 72 hours as measured by immunocytochemistry and qPCR. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are NNS pHBECs, open shapes are BEAS-2B cells.*

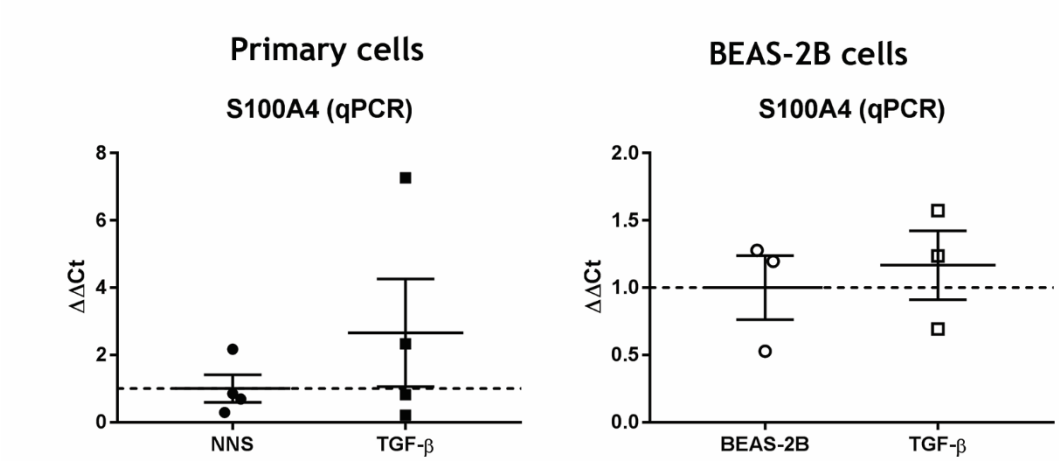

Figure 5.2-15: Expression of S100A4 in primary bronchial epithelial cells from non-smokers (NNS) and the immortalised BEAS-2B cell line following exposure to transforming growth factor-β1 (TGF-β) for 72 hours as measured by qPCR. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are NNS pHBECs, open shapes are BEAS-2B cells.*

5.2.2.3. Signalling molecules

TWIST expression in primary and BEAS-2B cells was unaffected by TGF-β at both the protein and mRNA level (Figure 5.2-16). , athlough it did not reach statistical significanceSimilarly, Smad6 was unaffected by TGF-β in both primaryand BEAS-2B cells (Figure 5.2-17). respondTGF-β appeared to have no effect on TWIST or Smad signalling in either primary or BEAS-2B cells.

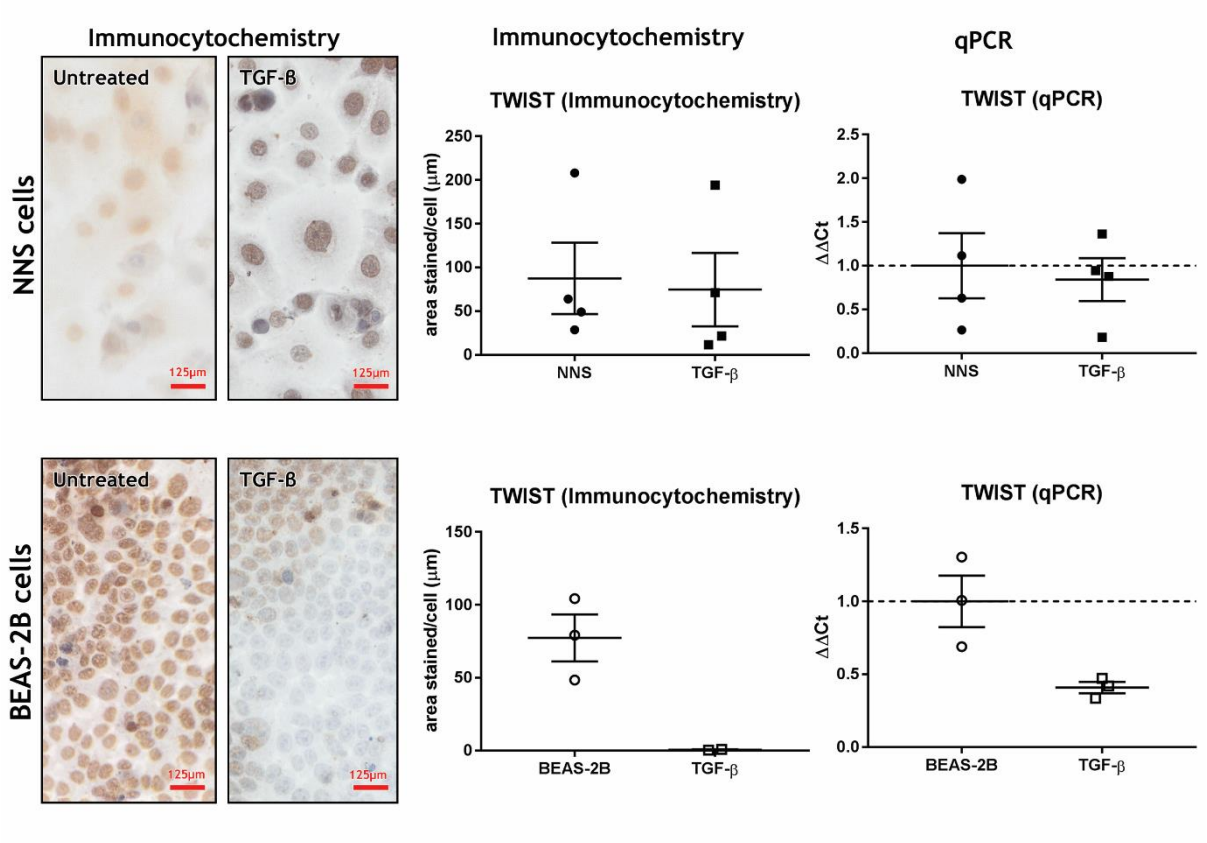

Figure 5.2-16: Expression of TWIST in primary bronchial epithelial cells from non-smokers (NNS) and the immortalised BEAS-2B cell line following exposure to transforming growth factor-β1 (TGF-β) for 72 hours as measured by immunocytochemistry and qPCR. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are NNS pHBECs, open shapes are BEAS-2B cells.*

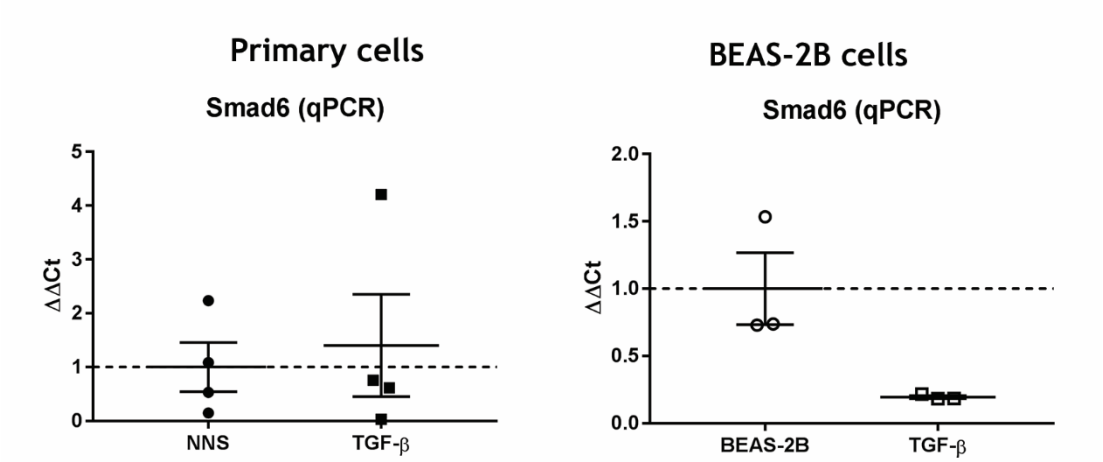

Figure 5.2-17: Expression of Smad6 in primary bronchial epithelial cells from non-smokers (NNS) and the immortalised BEAS-2B cell line following exposure to transforming growth factor-β1 (TGF-β) for 72 hours as measured by qPCR. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are NNS pHBECs, open shapes are BEAS-2B cells.*

5.2.2.4. Extracellular remodelling

Both primary cells and BEAS-2B cells demonstrated no change in MMP2 expression in response to TGF-β treatment (Figure 5.2-18), nornor did the expression of collagen 1-α change in response to the exposure. P proteinexpression in the primary cells also remaineded unchanged, however the BEAS-2B cells produced 15.1 times more protein following exposure (Welch's t-test p = 0.0158). Overall, TGF-β had littlelittle effect on extra-cellular matrix remodelling protein expression however, not unexpectedly, it produced an increase in pro-collagen 1-α expression, although only in the BEAS-2B cell line, while the primary cells remained unaffected by the exposure.

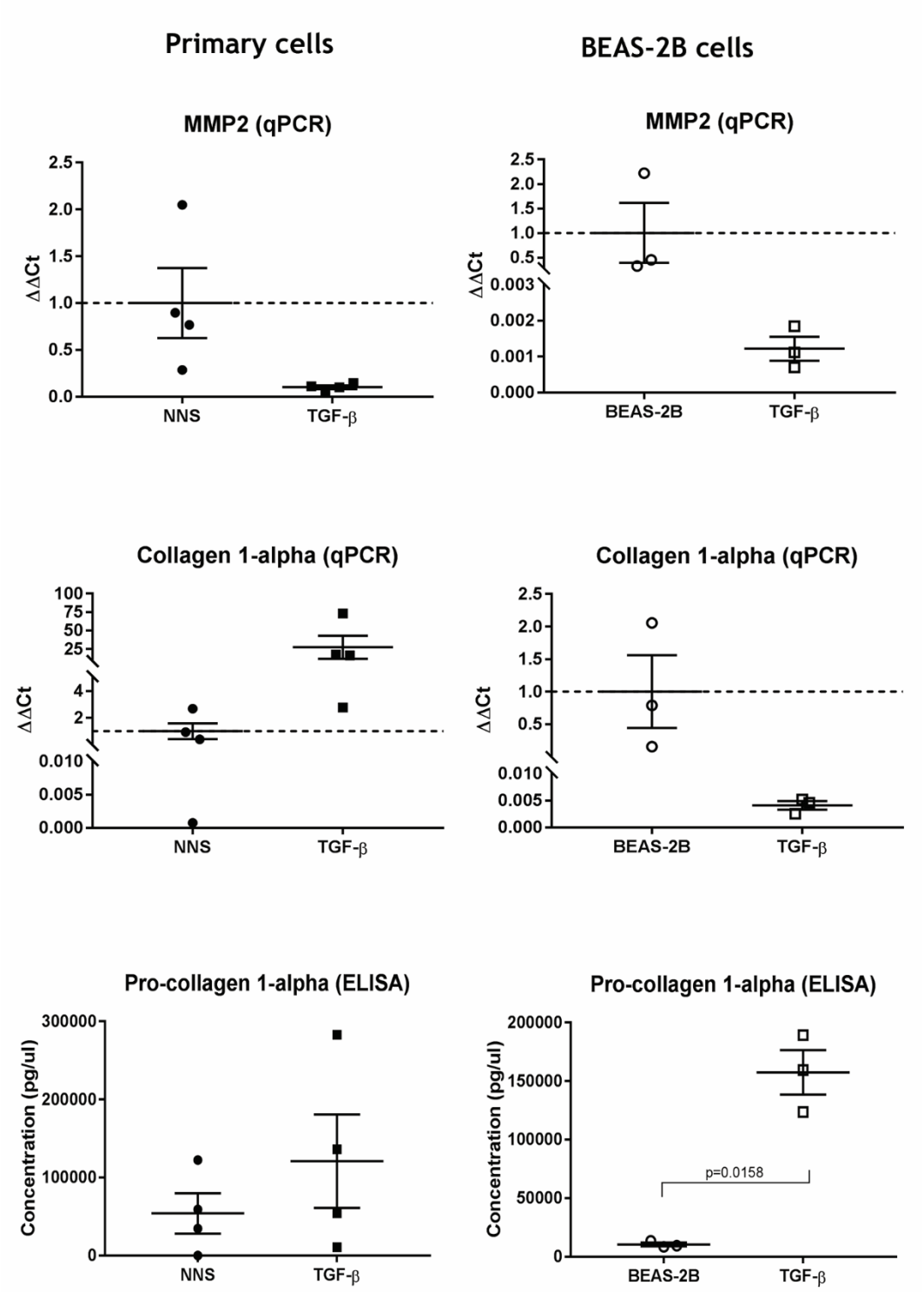

Figure 5.2-18: Expression of MMP2 and collagen 1-α in primary bronchial epithelial cells from non-smokers (NNS) and the immortalised BEAS-2B cell line following exposure to transforming growth factor-β1 (TGF-β) for 72 hours as measured by ELISA of cell culture supernatant and qPCR. *Data are represented as mean with SEM.* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are NNS pHBECs, open shapes are BEAS-2B cells.*

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

5.3. Chapter discussion

5.3.1. CSE discussion

Cigarette smoke extract (CSE) is an interesting substance to work with. Although it has clear biological relevance to smoking-related diseases such as chronic obstructive pulmonary disease (COPD), utilising it *in vitro* presents a unique challenge. Smokers vary in the frequency and depth of their puffs when smoking [268], and the chemical and particulate content of cigarette smoke varies between brands [263, 264]. These facts make it challenging to standardise the application of cigarette, although if a study focusses on a specific component such as nicotine they can standardise the content of that component [199]. In this study, the CSE used was kindly donated by others who had used the same extract in prior published work [209], and our colleague Jessica Kregor had previously performed dose response work in the author's group to find a concentration which was not lethal to BEAS-2B cells.

Primary cells reacted differently than BEAS-2B cells following exposure to cigarette smoke, with CSE exertingexerting a mixed effect on epithelial-mesenchymal transition (EMT) markers and pathway signalling molecules.. While CSE increasedincrease the mesenchymal profile of primary cells, it had no effect on epithelial markers, and actually decreased expression of the affected mesenchymal markers in BEAS-2B cells. theThis suggests that the BEAS-2B cell line is not ideal as a model of smoking-related changes in bronchial epithelial cells, and when possible primary cells should be employed instead.

With regards to the induction of EMT by CSE, the results were somewhat mixed, but generally suggested that CSE did not induce complete EMT in the cells. Epithelial markers

appeared unaffected, both in primary and BEAS-2B cells, however primary cells exhibited a significant increase in N-cadherin expression, although all other mesenchymal markers remained unchanged. While both primary and BEAS-2B cells exhibited a significant decrease in the level of pro-collagen 1-α in the culture supernatant, with levels too low to be detected, this may be due to the fact that the CSE-containing supernatant was only applied to the cells for 4 hours and therefore conained lower levels of secreted factors than the control supernatant, which was exposed to the cells for 24 hours. It would appear that CSE acts through neither the TWIST nor the Smad pathway, which disagrees with the literature [33], at least with regards to Smad. However, there are two possible explanations for this discrepancy – differences in the model system and differences in the markers measured.

Firstly, Milara and colleagues utilised a differentiated epithelium grown at an air-liquid interface, as compared to the submerged culture system used in the current study. This likely affected the responses of the cells to stimulation and may account in part for the observed difference.

The other fact is that Milara and colleagues measured activation of Smad3 expression, whereas the present study looked at Smad6 expression. Smad3 is part of the excitatory pathway in TGF-β signalling, being activated via phosphorylation and forming an active complex with Smad2, while Smad6 is an inhibitory Smad [87]. Smad6 is known to be upregulated in an auto-inhibitory feedback loop in response to TGF-β [87], which is upregulated in the airways of smokers [86, 240] and the expression of which is induced by CSE exposure in epithelial cells [33, 265], which makes it a good target for analysis via qPCR as compared to an activating Smad like Smad4, which is constitutively expressed [87]. However, it is possible that CSE, which contains many compounds and the composition of

which varies depending on the source, may act via an unknown mechanism to inhibit the production of inhibitory Smads, which would allow for the fact that Smad3 activation increased in the Milara paper while Smad6 expression remained unchanged in the current study. Another possibility is that CSE activates more strongly pathways such as the NF-κB pathway, which selectively upregulates the inhibitory Smad7 without Smad6 activation [87], although this theory does not account for the known activation of Smad3 seen in cells exposed to CSE [33].

Future study should look at both Smad6 expression and Smad3 phosphorylation in these cells, to attempt to disentangle the reason behind the conflicting results between this study and the literature. Analysis of the non-Smad associated MAPK pathway is also advisible, on the basis that TGF-β can also act via that mechanism [90]. Further, as with all chapters in this study, increased sample sizes are highly recommended to increase the power to detect changes in the level of expression of the markers.

Within the limitations of the current results, it is reasonable to conclude that the BEAS-2B cell line reacts differently to CSE than primary bronchial epithelial cells at the molecular level, although generally the protein-level responses appear similar. Thus, when the BEAS-2B cell line is used in place of primary cells, focus should be placed on protein analysis over molecular changes, as noted in previous chapters. Additionally, while CSE appeared to induce a partial EMT phenotype in the primary cells it did not appear to be a potent inducer of EMT in the BEAS-2B cells, even being slightly suppressive, and thus primary cells should be preferred when examining the effects of CSE on bronchial epithelial cells in culture, particularly when examining mRNA level changes.

5.3.2. TGF-β discussion

In this study, exposure to transforming growth factor-β1 (TGF-β) failed to induce signs of epithelial-mesenchymal transition in primary cells taken from non-smokers or in BEAS-2B cells, although it did trigger increased expression of collagen 1-α in BEAS-2B cells. While the fact that the primary and BEAS-2B demonstrated similar responses to TGF-β suggests that BEAS-2B cells may be a useful model of healthy bronchial epithelial cells' reactions to cytokines, the fact that TGF-β exposure failed to induce EMT in either cell type is a noticeable issue.

The literature demonstrates, in epithelial cells taken from multiple different origins, that TGF- β is a reliable inductor of EMT [33, 63, 92, 93, 165, 200, 257-259, 266], which raisedd the question of why it failed to produce an EMT phenotype in the current study. Previous work, during the author's Honours work and by others within the group with TGF-β in the BEAS-2B cell line has generated data consistent with the literature, making this non-result a curiosity. This reduced activity is worth noting, however the TGF-β still retained some activity, as evidenced by the increase in pro-collagen 1-α expression in the BEAS-2B cells the cytokine had not lost all activity, and thus may still yield some useful data in future experiments.

Repetition of these experiments with fresh cytokine would be recommended in order to either validate the unresponsiveness of the cells to TGF-β or, more likely, to properly examine the differences in response of the primary cells and the BEAS-2B cells to TGF-β. Furthermore, prior to experimentation it is recommended to test the activity of the TGF-β cytokine, which can be done utilising the MBF-11 or TMLC cell line-based bioassayslines [267, 268].

With the current results, it is possible to speculate that the primary cells and BEAS-2B cells may respond differentially to TGF-β along the TWIST and Smad pathways, and that the BEAS-2B cell line may have a more extreme pro-fibrotic response to the cytokine than the primary cells. This data suggests that while the BEAS-2B cells may be useful substitutes for primary cells with regards to EMT markers, they are unreliable models of pathway mechanisms and fibrotic changes.

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

5.4. Chapter conclusions

As in previous chapters, primary and BEAS-2B cells differed in their molecular level expression of markers and other factors, in this case following exposure to transforming growth factor-β1 (TGF-β) or cigarette smoke extract (CSE). Although they generally agreed at the protein level of expression, it would appear preferable to avoid using the BEAS-2B cell line to represent healthy bronchial epithelial cells whenever possible, and if it cannot be avoided then protein-level data should be preferred over mRNA expression.

In this study, despite the literature, neither TGF-β nor CSE appeared to successfully induce a full EMT phenotype in either primary or BEAS-2B cells. Further study with fresh cytokine and, if possible, a larger number of samples to confirm or deny possible changes observed in this study is highly recommended.

Useful abbreviations

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TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

6. Effect of current COPD therapeutic drugs on EMT *in vitro*

6.1. Chapter aims

The aims of this chapter were as follows:

- To examine the effects of the chronic obstructive pulmonary disease drugs salmeterol xinafoate, tiotropium bromidebormide monohydrate and fluticasone proprionate, both alone and in combination on epithelial-mesenchymal transition (EMT) in epithelial cells taken from non-smokers, smokers with normal lung function and people with airflow obstruction, as well as in the immortalised BEAS-2B cell line.
    - **Hypothesis:** Salmeterol xinafoate will suppress EMT markers, possibly by interaction with TWIST. Tiotropium bromide monohydrate will suppress EMT related markers via suppression of the TGF-β signalling pathway, potentially via Smad signalling. Fluticasone proprionate will decrease EMT related markers, although the potential mechanisms behind this are unclear.
- To examine the effects of the three drugs alone and in combination on EMT in both normal primary epithelial cells taken from non-smokers and in the immortalised BEAS-2B cell line following application of either transforming growth factor-β1 or cigarette smoke extract.

    - **Hypothesis:** Salmeterol xinafoate will suppress EMT changes by interaction with TWIST, with best effect on those changes induced by cigarette smoke

extract. Tiotropium bromide monohydrate will suppress EMT related changes via the , especially those induced by transforming growth factor-β1 signalling pathway.. Fluticasone proprionate will decrease EMT related changes and will be effective on EMT induced by either transforming growth factor-β or cigarette smoke extract.

The aims of this section were as follows:

- To examine the effects of combined salmeterol, tiotropium and fluticasone on EMT markers, two signalling pathway markers and two extracellular matrix remodelling factors in primary bronchial epithelial cells taken from non-smokers, smokers with normal lung function and people with COPD, as well as in the commercially available immortalised BEAS-2B cell line.
    - **Hypothesis:** The combination of the three drugs will exert a synergistic EMT-suppressing effect on both primary and immortalised bronchial epithelial cells.cells. The drugs will also suppress both the TWIST and Smad signalling pathways.
- To examine the effects of either transforming growth factor-β1 or cigarette smoke extract combined with salmeterol, tiotropium and fluticasone on EMT markers, two signalling pathway markers and two extracellular matrix remodelling factors in primary bronchial epithelial cells taken from non-smokers and the commercially available immortalised BEAS-2B cell line.
    - **Hypothesis:** The combination of the three drugs will suppress EMT-related changes caused by exposure to either transforming growth factor-β1 or cigarette

smoke extract. The drugs will also suppress both the TWIST and Smad signalling pathways.

Useful abbreviations

NNS *– non-smokers*
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COPD-CS *– current smokers with airflow limitation*
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6.2. Salmeterol xinafoate

6.2.1. Introduction

Salmeterol is a long-acting beta-2 adrenoreceptor-agonist (LABA) used in the treatment of asthma and COPD [269]. It is lipophilic and accumulates in the cell membrane, which allows it to act over a longer period of time than short-acting bronchodilators (SABAs) [270]. However, because it is a partial agonist, compared to a full agonist like formoterol, it has greatly reduced functionality in inflamed tissue, and unlike formoterol the concurrent application of a corticosteroid does not improve this reduced effect. Despite this, salmeterol a common apharmacological therapyy used to treat COPD [208].

Salmeterol and other LABAs reduce inflammation in the airways[270] as well as reducing airway remodelling and slowing the decline in lung function which results from COPD [271, 272]. They are commonly prescribed in COPD, and have been shown to be effective at increasing quality of life, as well as reducing the number of exacerbations [271, 273, 274]. LABAs like salmeterol act via binding to β2-adrenergic receptors, which are known to be present on airway epithelial cells [111, 173, 233, 234, 272, 275, 276], and cause upregulation of cAMP and the MAPK pathways in order to reduce inflammation [173, 234, 275]. There are a number of long-term effects which come from prolonged use of LABAs, including desensitisation, increased inflammatory responses and mucin (mucous proteins) production [234, 275], however LABAs, along with long acting muscarinic antagonists (LAMAs) are among the few non-surgical therapies which are known to slow the progression of COPD [271].

Of specific interest to this study, LABAs are known to reduce epithelial cells' motility and impair wound healing [272], which suggests that it may be having an effect on the process of

EMT. It is therefore possible that some of salmeterol's effects in COPD may be due not only to its effects on the inflammatory lung environment, but also changes it is capable of producing in epithelial cells. Examining the effect of salmeterol on EMT, which is thought to cause airway remodelling, may shed further light on salmeterol's actions in COPD and elucidate the pathway by which it acts to slow or prevent fibrosis.

Useful abbreviations

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6.2.2. Results

6.2.2.1. Effects of salmeterol on EMT markers in primary and BEAS-2B cells

6.2.2.1.1. Epithelial markers

Salmeterol had no effect on E-cadherin expression at the protein level or mRNA in primary cells (Figure 6.2-1). BEAS-2B cells' expression of E-cadherin waslikewise unaffected by application of salmeterol. None of the three primary cell groups exhibited any change in cytokeratin protein expression in response to salmeterol, however BEAS-2B cells increased protein expression by 1.9 times (one-way ANOVA p = 0.0278) following exposure to salmeterol (Figure 6.2-2). NeitherNeither primary cells nor BEAS-2B cells exhibited any change in tight junction-1 (TJP1) mRNA expression in response to salmeterol (Figure 6.2-3). Overall, salmeterol had nono effect on epithelial junction expression, however increased expression of cytokeratine in BEAS-2B cells2 cells.

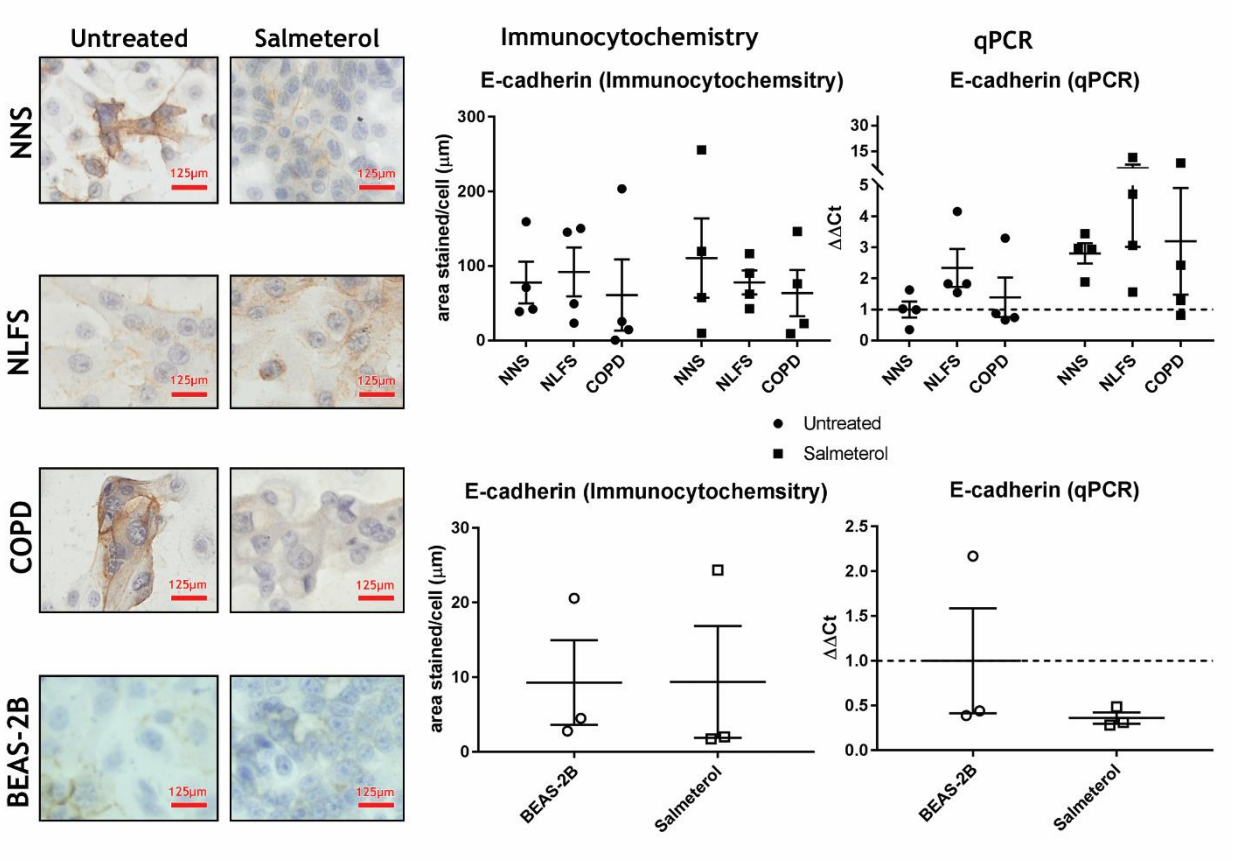

Figure 6.2-1: Expression of E-cadherin in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to salmeterol for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

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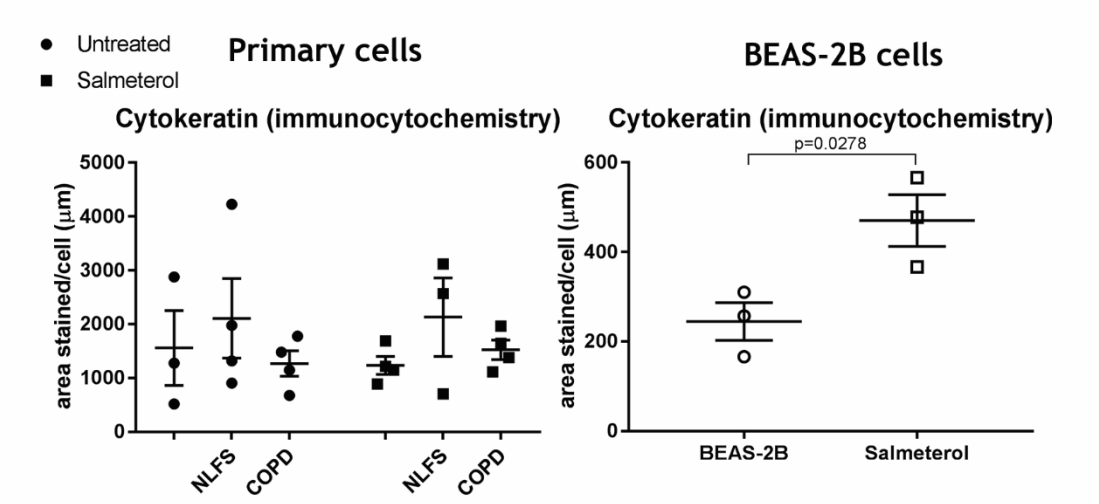

Figure 6.2-2: Expression of cytokeratin in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by immunocytochemistry with and without exposure to salmeterol for 24 hours. *Data are represented as mean with SEM. In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

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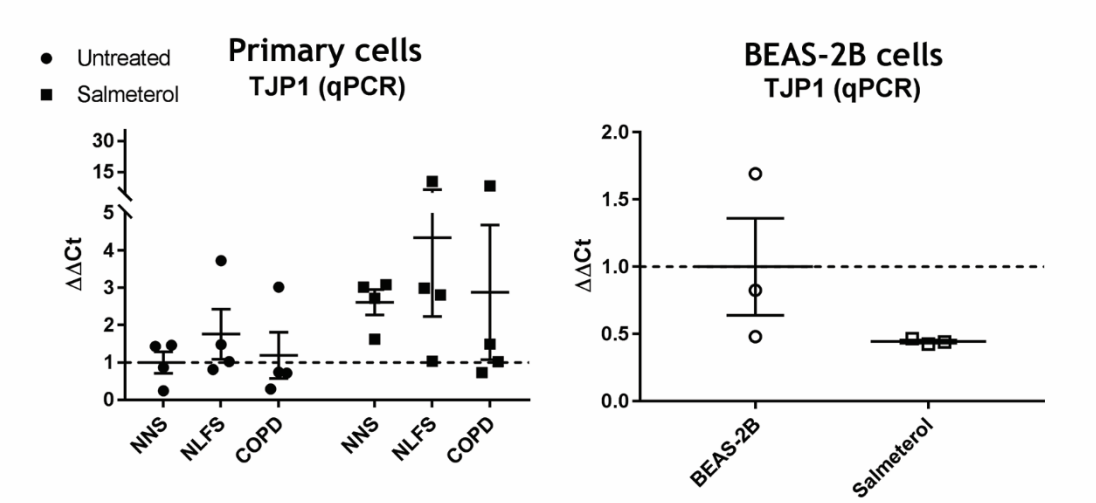

Figure 6.2-3: Expression of tight junction protein-1 (TJP1) in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by qPCR with and without exposure to salmeterol for 24 hours. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

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pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

6.2.2.1.2. Mesenchymal markers

At the protein level, salmeterol reduced variatbilityreduce variation in N-cadherin protein expression in cells from smokers with normal lung function, although this change couldn't be analysed statistically, however N-cadherin expression in the other primary groups was unchanged (Figure 6.2-4). At the mRNA level there was no change in N-cadherin expression in any of the three primary cell groups, and at both the protein and mRNA level N-cadherin expression in the BEAS-2B cells appeared unchangedunchanged.

Vimentin expression appeared broadly unchanged by salmeterol treatment in both primary and BEAS-2B cells, although primary cells taken from smokers with normal lung function exhibited reduced variation in protein expression following treatment, which again could not be quantified statistically (Figure 6.2-5). Likewisewith the exception of a couple of samples in the COPD-affected group which increased expression of S100A4 mRNA, salmeterol had no effect on either primary or BEAS-2B cells' expression of S100A4 (Figure 6.2-6).

Overall, while salmeterol appeared to reduce variation in mesenchymal marker expression in smokers with normal lung function, the drug had no other effect on mesenchymal marker expression in either primary or BEAS-2B cells.

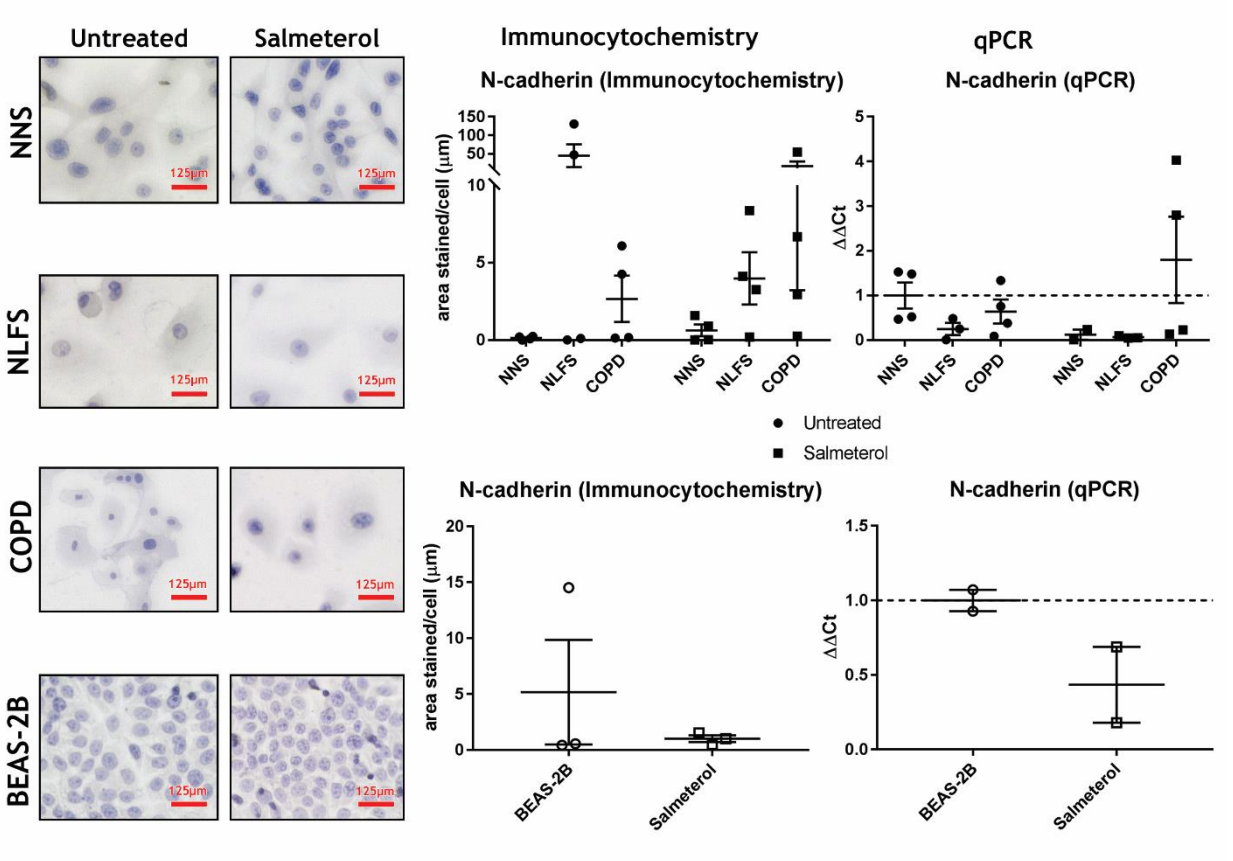

Figure 6.2-4: Expression of N-cadherin in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to salmeterol for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

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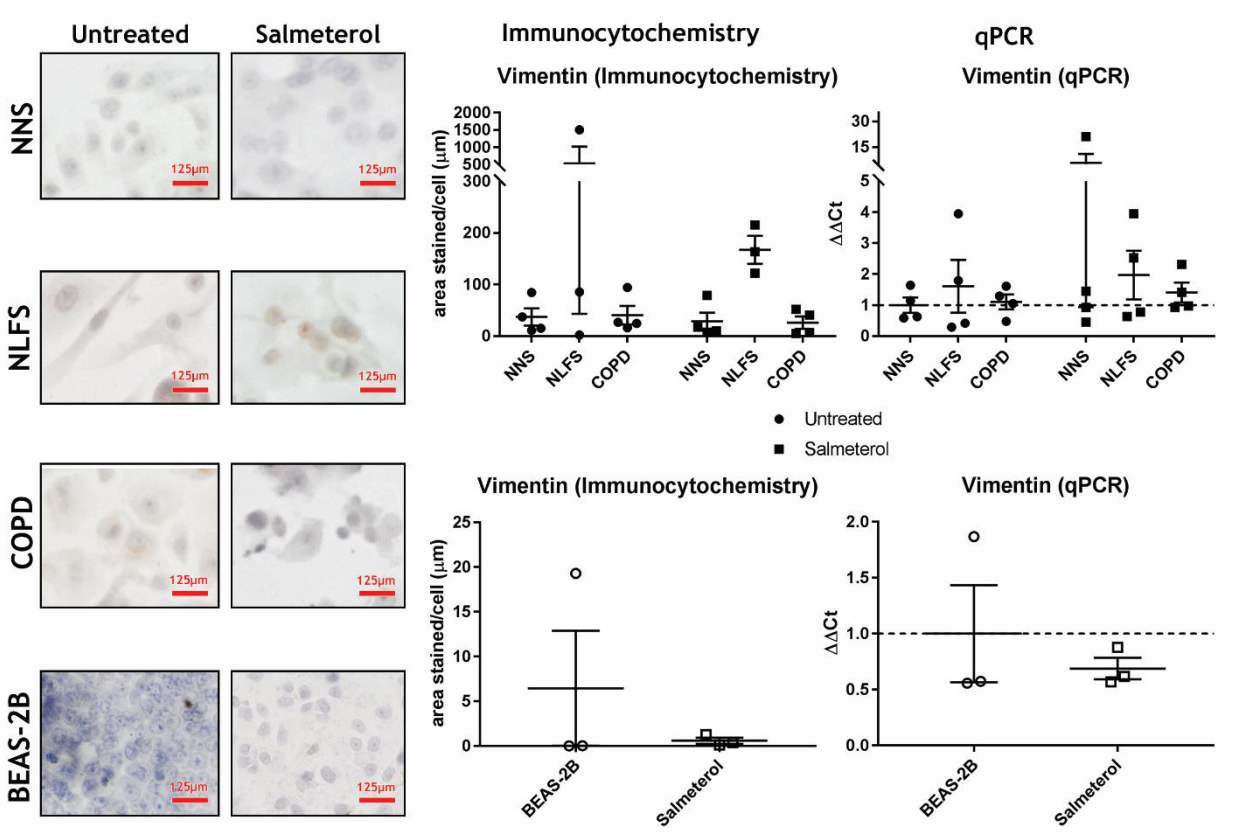

Figure 6.2-5: Expression of vimentin in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to salmeterol for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

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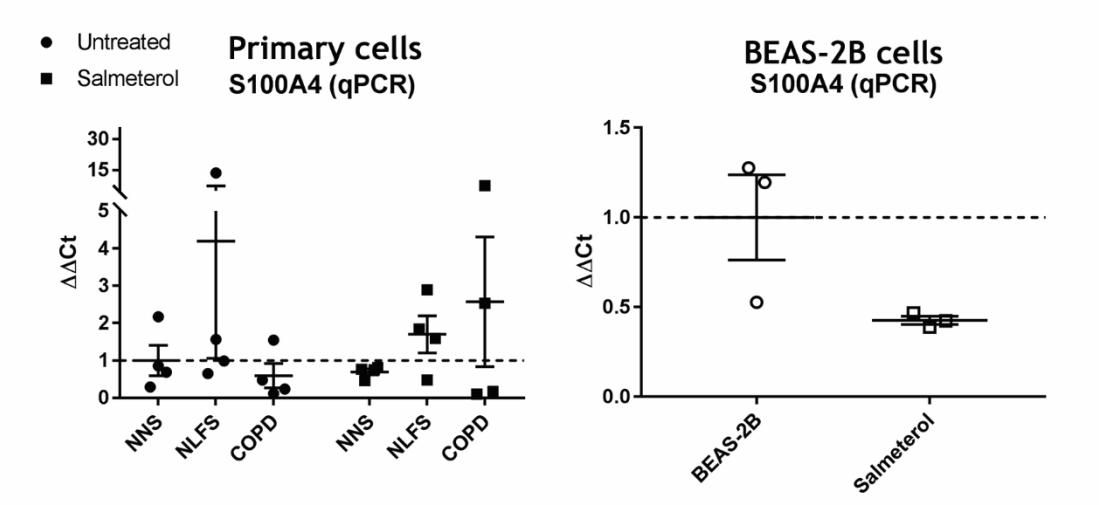

Figure 6.2-6: Expression of S100A4 in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by qPCR with and without exposure to salmeterol for 24 hours. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

6.2.2.1.3. Signalling molecules

Exposure to salmeterol appeared to havehave no effect on TWIST mRNA expression in both primary and BEAS-2B cells (Figure 4.2-7). Smad6 expression waswas likewise unaffected by exposure to salmeterol (Figure 6.2-8). Overall, salmeterol hadhave no effect on either TWIST or Smad6 expression.

Useful abbreviations

NNS *– non-smokers*
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COPD-ES *– ex-smokers with airflow limitation*
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CSE *– cigarette smoke extract*

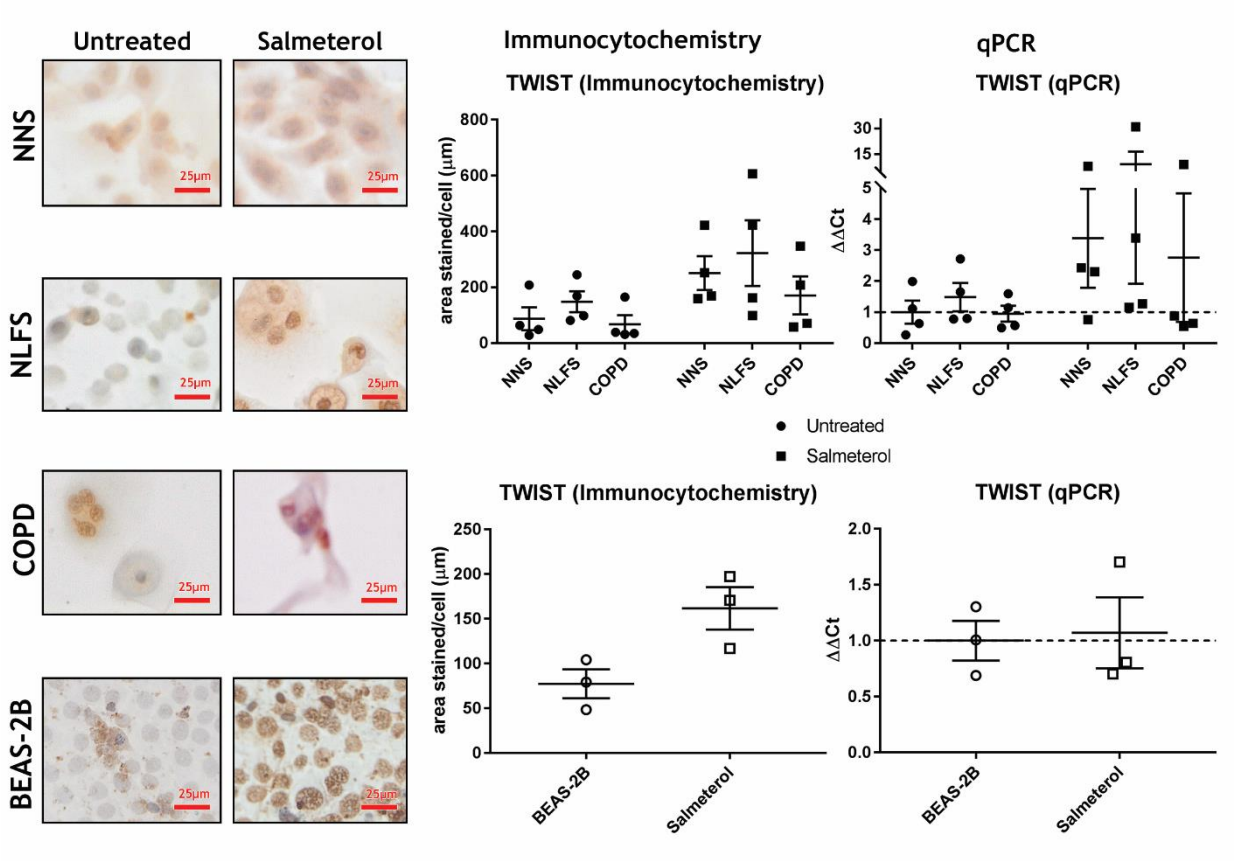

Figure 6.2-7: Expression of TWIST in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD)people with airflow limitation (COPD) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to salmeterol for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells. ΔΔCT shows the fold-change in expression, with '1' being no change from baseline or the control.*

Key: *Filled shapes are pHBECs, open shapes are BEAS-2B cells.*

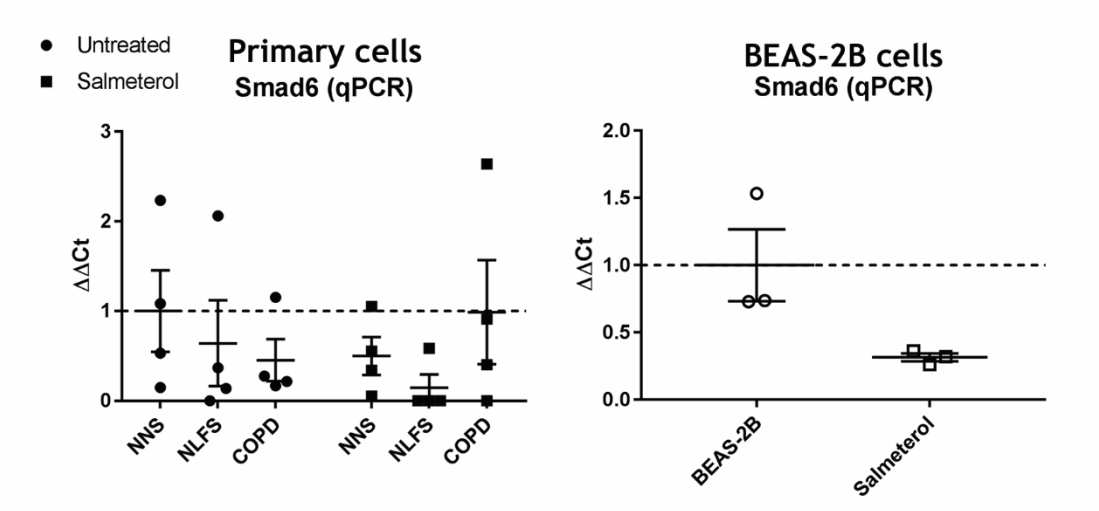

Figure 6.2-8: Expression of Smad6 in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by qPCR with and without exposure to salmeterol for 24 hours. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

Useful abbreviations

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COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

6.2.2.1.4. Extracellular matrix remodelling

Across all three groups, primary cells were unaffected by exposure to salmeterol with regards to both MMP2 and collagen 1-α expression, a result which waswas mirrored by the BEAS-2B cells (Figure 6.2-9).

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

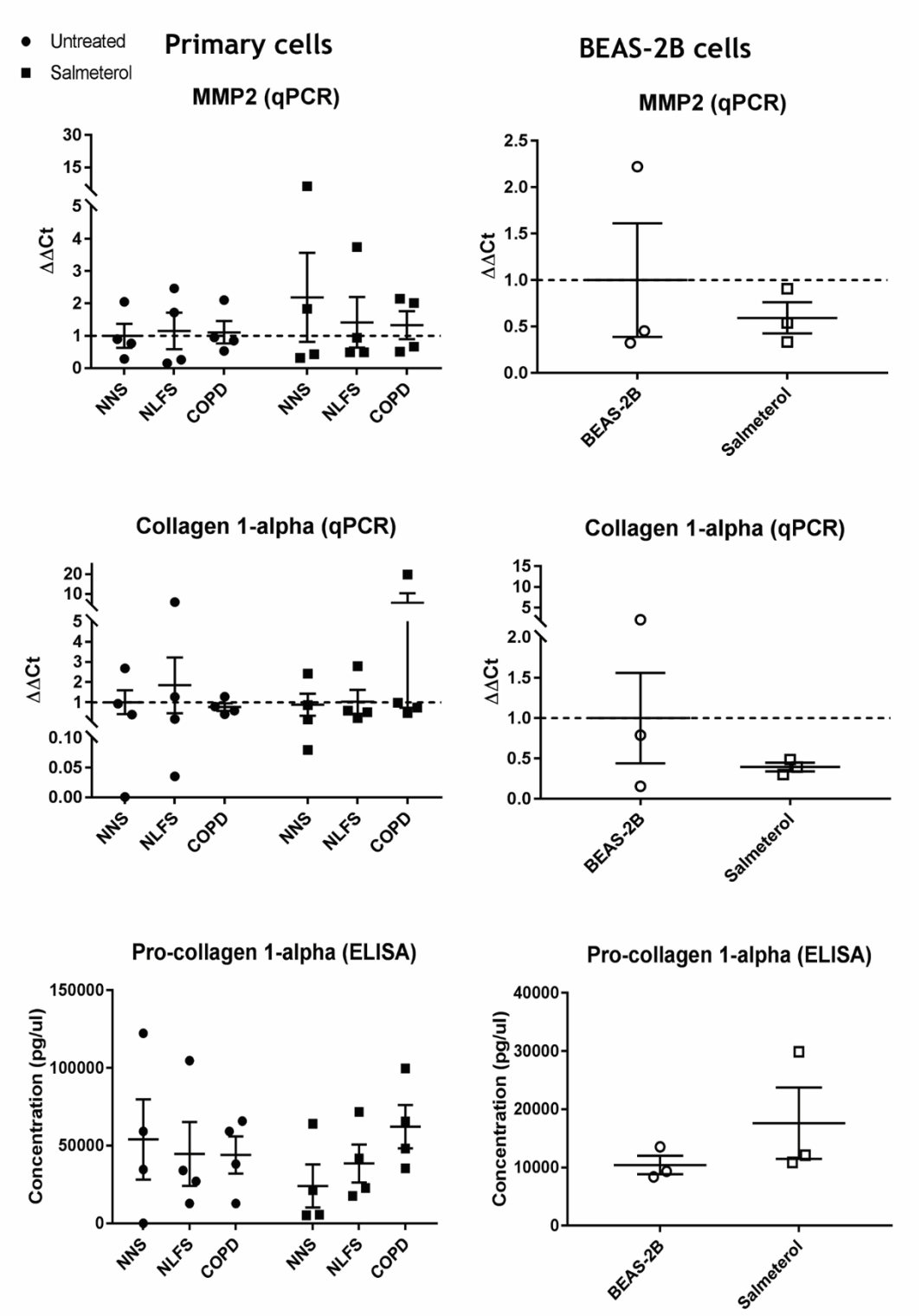

Figure 6.2-9: Expression of MMP2 and collagen 1-α in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by ELISA of cell culture supernatant and qPCR with and without exposure to salmeterol for 24 hours. *Data are represented as mean with SEM.* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

Useful abbreviations

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COPD-ES *– ex-smokers with airflow limitation*
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pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

6.2.2.2. TGF-β and CSE-induced EMT

6.2.2.2.1. Epithelial markers

In both primary and BEAS-2B cells treated with transforming growth factor-β1 (TGF-β), salmeterol had no effect overall on E-cadherin expression at either the protein or mRNA level (Figure 6.2-10). CSE appeared to increase variability with the primary cell grousp, although this could not be tested statisitcally and the cells' average mRNA levels remained unaffected.

The combination of TGF-β and salmeterol had no effect on cytokeratin protein expression in primary cells, however increased protein expression 3.7-fold (one-way ANOVA p = 0.0090) in BEAS-2B cells (Figure 6.2-11). The combination of salmeterol with CSE had no effect on cytokeratin expression in either primary or BEAS-2B cells.

Tight junction protein-1 (TJP1) expression in primary and BEAS-2B cells was unaffected following treatment with TGF-β and salmeterol (Figure 6.2-12). However, in both primary and BEAS-2B cells the combination of TGF-β and salmeterolcalmeterol did reduceappear to reduce expression of TJP1 in cells which initially expressed high levelsit more highly initially, although this cannot be statistically confirmed.

Overall, the combination of TGF-β and salmeterol had no effect on epithelial markers in primary cells, although the combination increased cytokeratin expression compared to TGF-β treated cells in the BEAS-2B cell line. CSE and salmeterol appeared to suppress expression of TJP1TJP1 in previously highly expressing samples, however this was not confirmed via statistical analysisanalysis.

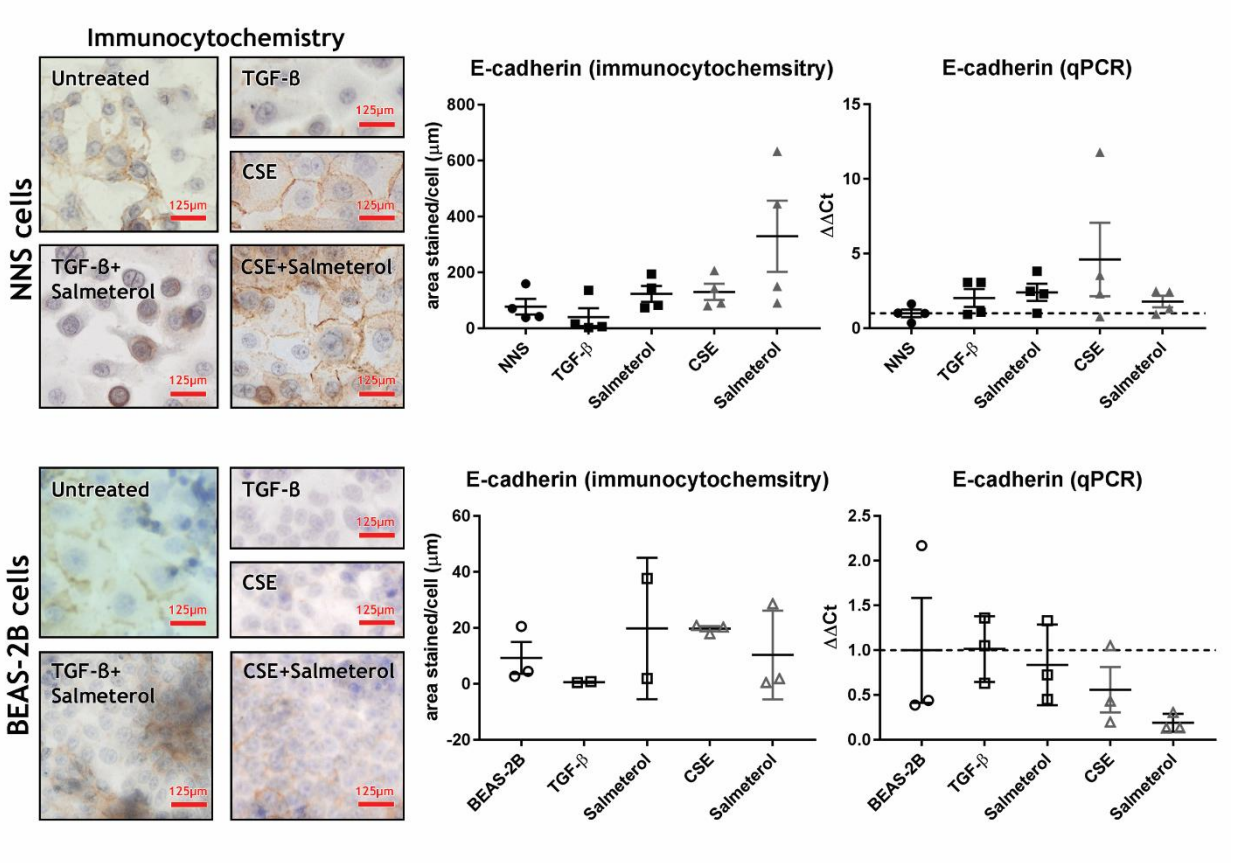

Figure 6.2-10: Expression of E-cadherin in non-smokers (NNS) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with salmeterol for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

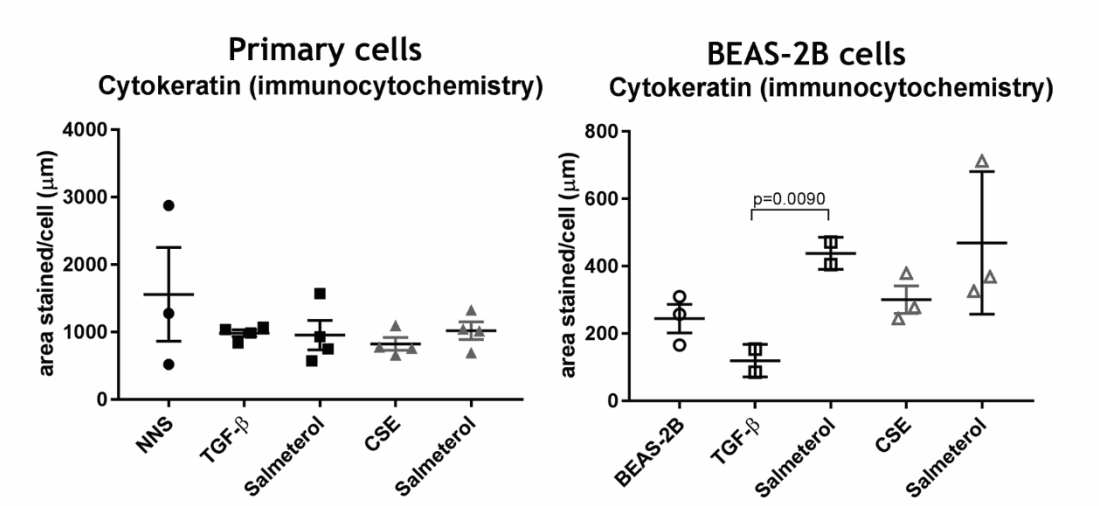

Figure 6.2-11: Expression of cytokeratin in non-smokers (NNS) and immortalised BEAS-2B cells as measured by immunocytochemistry with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with salmeterol for 24 hours. *Data are represented as mean with SEM. In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

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COPD-CS *– current smokers with airflow limitation*
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LABA/LAMA – *long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

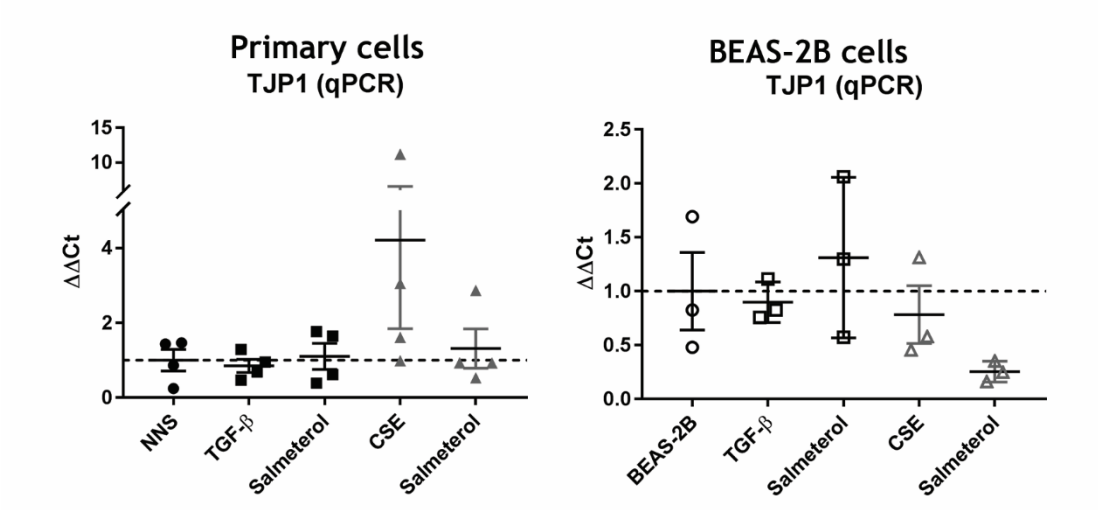

Figure 6.2-12: Expression of tight junction protein-1 (TJP1) in non-smokers (NNS) and immortalised BEAS-2B cells as measured by qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with salmeterol for 24 hours. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

6.2.2.2.2. Mesenchymal markers

Salmeterol applied to cells treated with transforming growth factor-β1 (TGF-β) did notaffect N-cadherin expression in primary cells or BEAS-2B cells at either the protein or mRNA level in BEAS-2 cells (Figure 6.2-13).While salmeterol appeared to rescuedid technically 'rescue' the change in N-cadherin mRNA expression in primary cells caused by CSE, based on the statistical analysis, the salmeterol-treated cells were not significantly different in expression compared to the cells treated with CSE alone and the change is likely due to increased variability between samples rather than being a true change.. In primary cells, cigarette smoke extract (CSE) and salmeterol in combination had no effect on N-cadherin protein or mRNA expression compared to CSE alone. CSE and salmeterol had no effect on protein level expression of E-cadherin compared to CSE alone in BEAS-2B cells, although only a single datum point was available for mRNA work, which meantt no conclusions could be drawn on that front.

Salmeterol hadto no effect on changes in vimentin expression caused by TGF-β in either primary or BEAS-2B cells when looking at the protein and mRNA expression (Figure 6.2-14). Similarly, salmeterol and TGF-β combined hadhave no effect on primary or BEAS-2B cells (Figure 6.2-15). In both primary and BEAS-2B cells treated with CSE, the addition of salmeterol di dnothad no effect on S100A4 expression.

Overall, salmeterol had no effect on TGF-β induced changes in mesenchymal markers in either primary cells or the BEAS-2B cell line. , possibly decreasing,

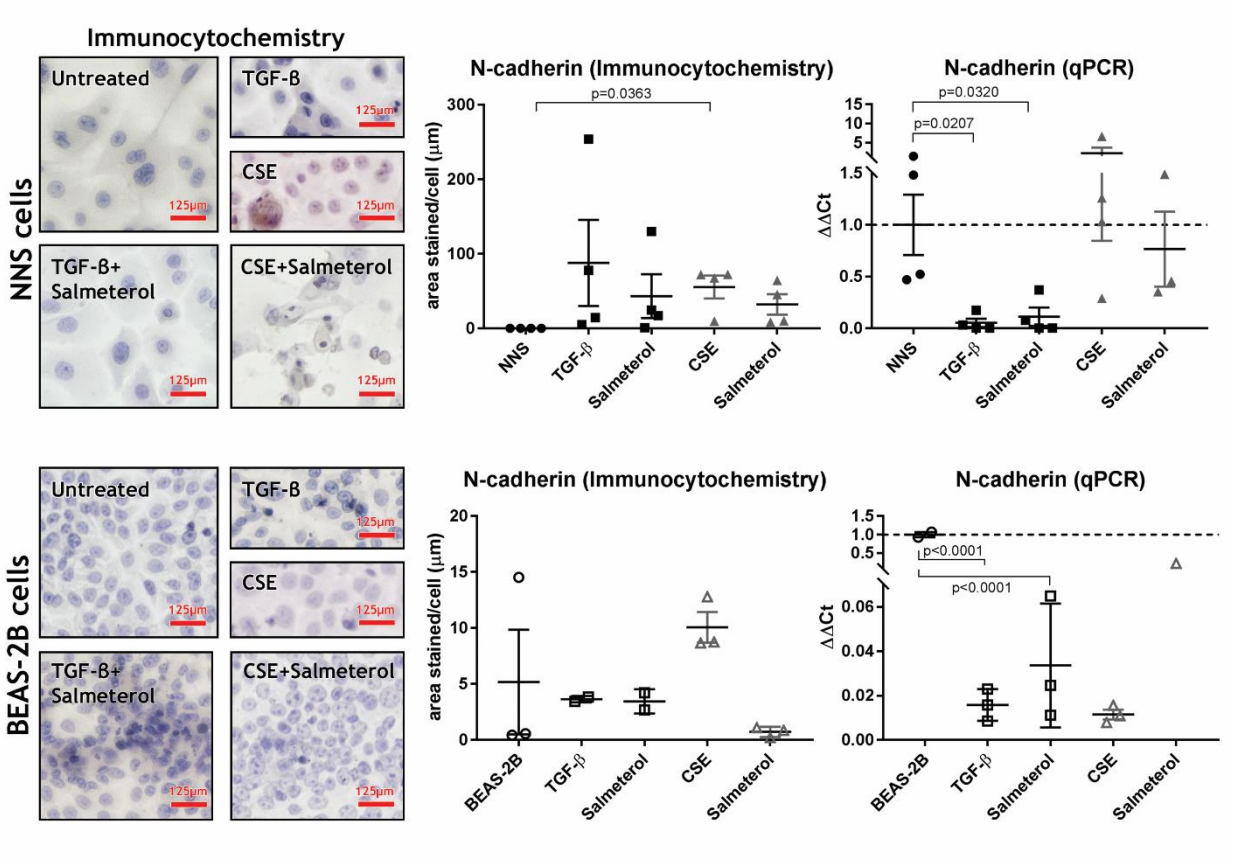

Figure 6.2-13: Expression of N-cadherin in non-smokers (NNS) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with salmeterol for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

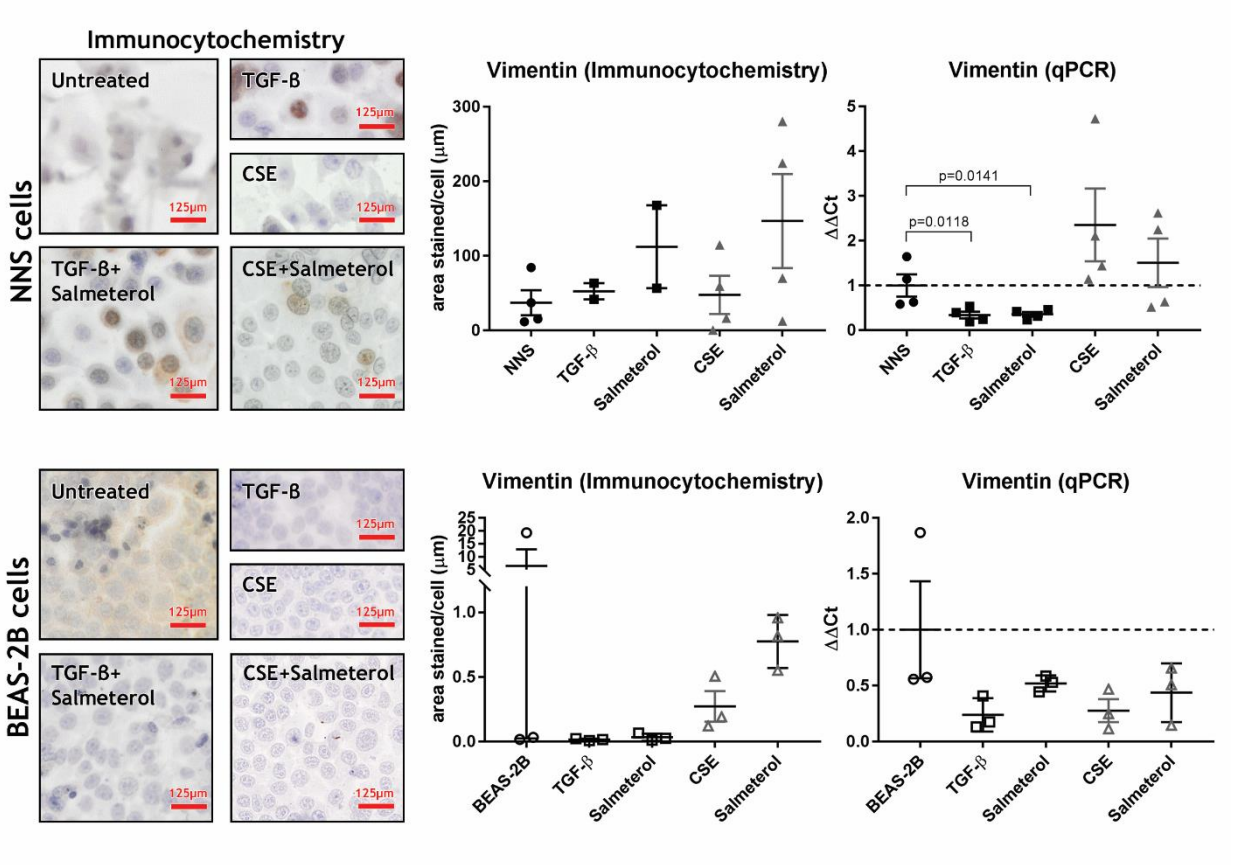

Figure 6.2-14: Expression of vimentin in non-smokers (NNS) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with salmeterol for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

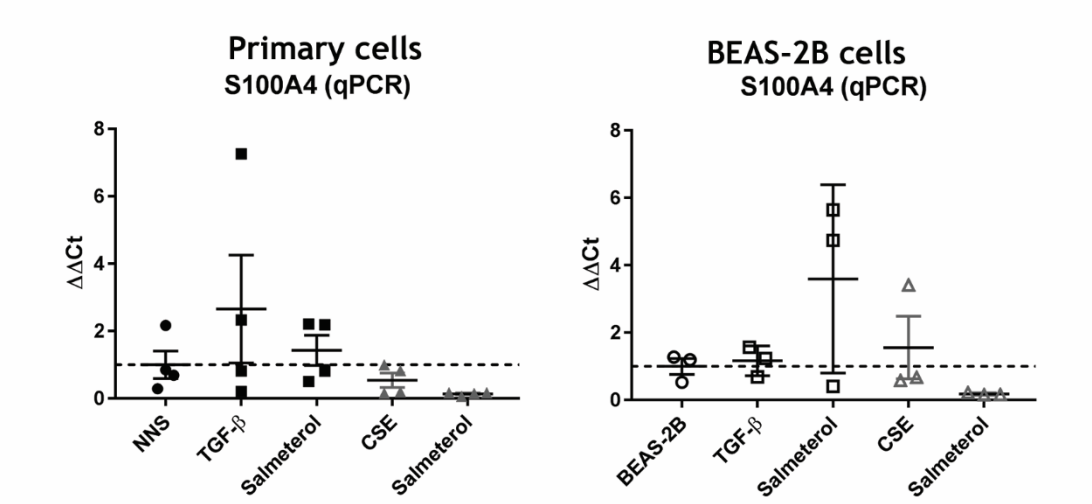

Figure 6.2-15: Expression of S100A4 in non-smokers (NNS) and immortalised BEAS-2B cells as measured by qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with salmeterol for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

6.2.2.2.3. Signalling molecules

At the protein level in both primary and BEAS-2B cells, the combination of salmeterol and transforming growth factor-β1 (TGF-β) or salmeterol and CSE had no effect on the expression of TWIST in either primary or BEAS-2B cells (Figure 6.2-16). However, CSE and salmeterol in combination increased expression of TWIST in BEAS-2B cells 3.2-fold (one-way ANOVA p = 0.0323) at the mRNA level, while in the primary cells it was unchanged, although there waswith a non-statisitcally significant trend towards reduced expressionsuggestion of a possible decrease in expression.

Smad6 mRNA expression was unaffected by the combination of salmeterol and either TGF-β or CSE in both primary cells and BEAS-2B cells (Figure 6.2-17).Overall, salmeterol appeared to effect BEAS-2B cells and primary cells treated with CSE differently when looking at thethe TWIST signalling pathway, although the drug had no effect on Smad signalling in either treatment group.

Useful abbreviations

NNS *– non-smokers*
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COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
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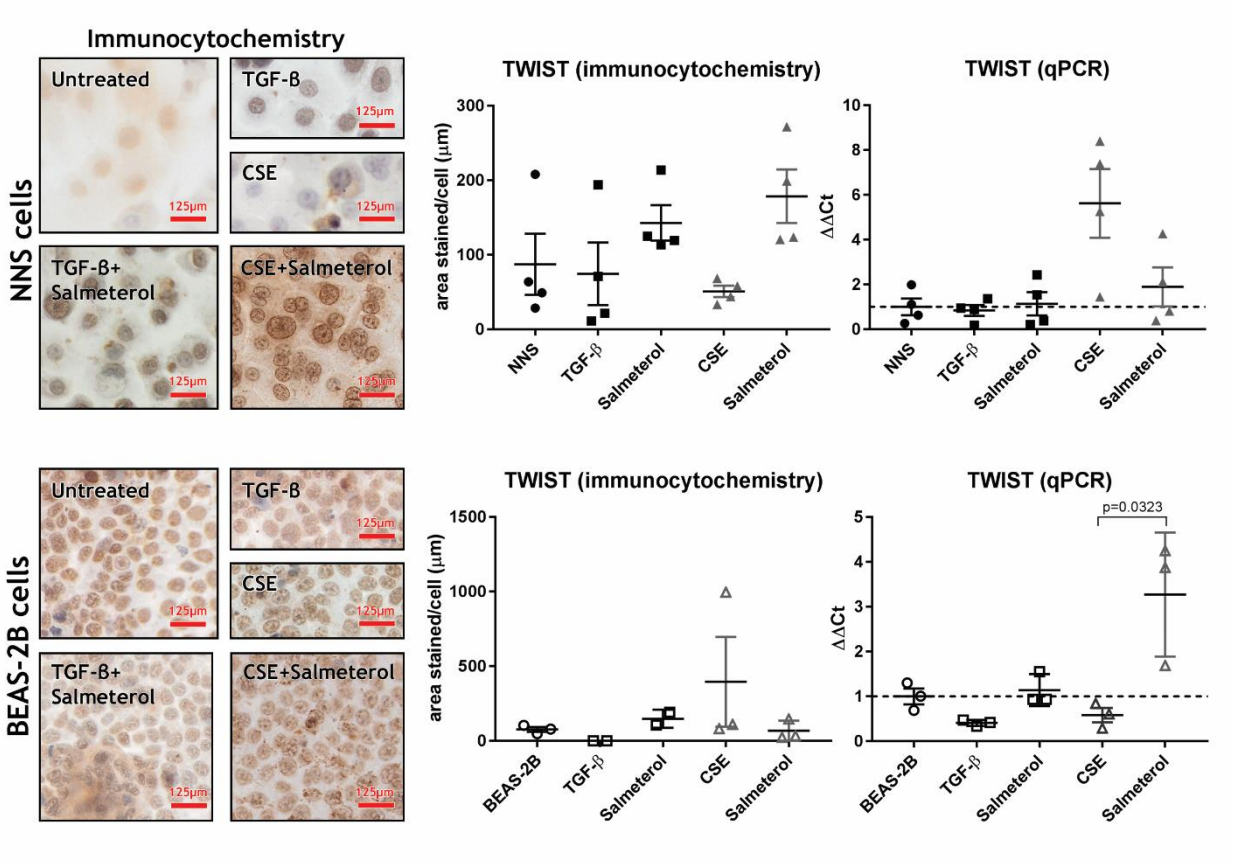

Figure 6.2-16: Expression of TWIST in non-smokers (NNS) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with salmeterol for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

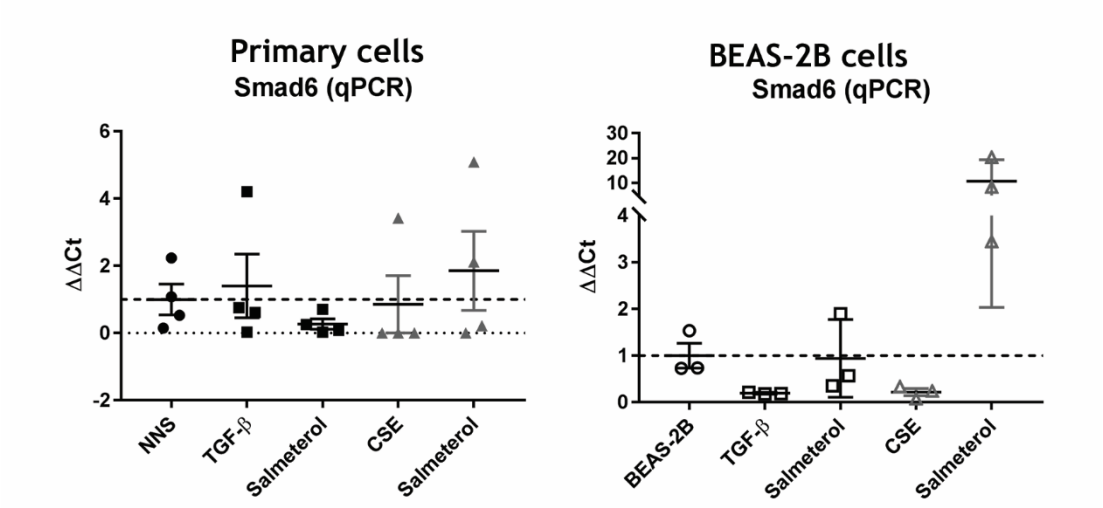

Figure 6.2-17: Expression of Smad6 in non-smokers (NNS) and immortalised BEAS-2B cells as measured by qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with salmeterol for 24 hours. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

6.2.2.2.4. Extracellular matrix remodelling

Salmeterol had no effect on TGF-β induced changes in either MMP2 or collagen-1α expression in primary or BEAS-2B cells, nor did it affect pro-collagen 1-α expression in either cell group when the cells were treated with either TGF-β or CSE (Figure 6.2-18). Likewise, salmeterol hadappeared to have no effect on MMP2 or collagen 1-α expression in primary or BEAS-2B cells treated with CSE. appeared to possibly haveisOverall, salmeterol had no effect on fibrotic changes caused by either TGF-β or CSE.

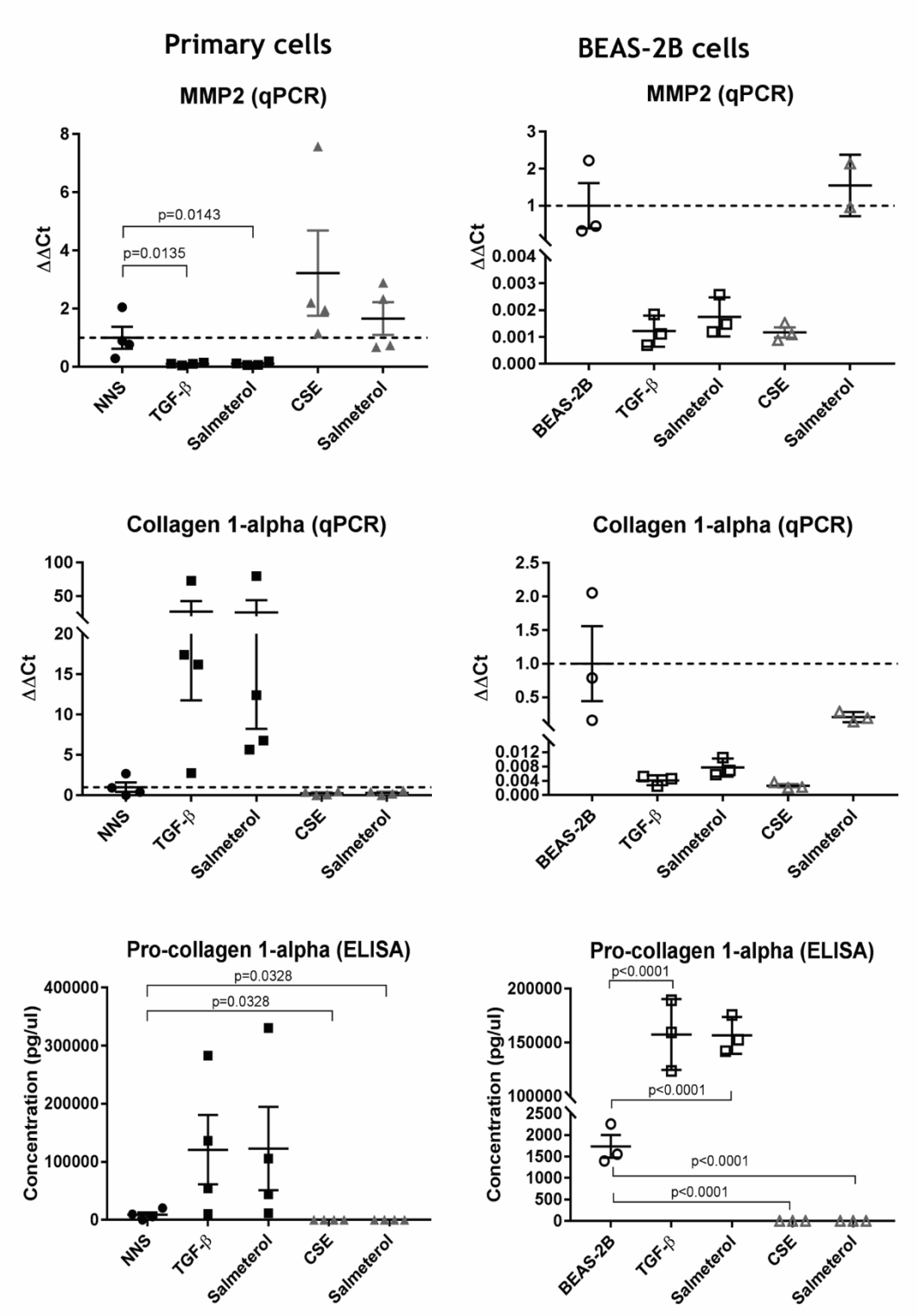

Figure 6.2-18: Expression of collagen 1-α and MMP2 in non-smokers (NNS) and immortalised BEAS-2B cells as measured by ELISA of cell culture supernatant and qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with salmeterol for 24 hours. At both the protein and mRNA levels, treatment with salmeterol fails to rescue the effects of either TGF-β or CSE on collagen 1-α expression in primary cells and has no effect on MMP2 expression in the same. *Data are represented as mean with SEM.* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

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TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

6.2.3. Discussion of the effects of salmeterol

Primary cells and BEAS-2B cells in this study reacted differently to salmeterol, indicating that BEAS-2B cells may not be a reliable model of healthy bronchial epithelial cells from non-smokers. These differences were most apparent at the mRNA level, and once again support the suggestion that when utilising BEAS-2B cells as a model of healthy primary epithelial cells, focus should be placed on protein-level data as opposed to mRNA level data in order to get more accurate representation of what occurs in primary cells.

In terms of its effects, salmeterol , increased expression of cytokeratin in BEAS-2B cellsS100A4, while exerting no effect on other epithelial markers, extracellular matrix proteins or mesenchymal markers in either cell type.. In both primary cells and BEAS-2B cells treated with transforming growth factor-β (TGF-β) salmeterol appeared to exert no additional effect on EMT-related markers, however in BEAS-2B cells exposed to cigarette smoke extract (CSE) there a trend towards a suppressive effect of salmeterol, although it did not reach statistical significance. appeared The lack of statistical significance may in part be attributable to large variation between samples within the groups, and if these experiments are repeated then it would be recommended to synchronise the cell cycle of the samples via serum starvation to reduce this variation. Samples were synchronised by apparent confluence for this study, however extra steps should be taken in future to further reduce variation.

Salmeterol alonealone did not affect TWIST signalling, which was unexpected given that salmeterol acts via upregulating cAMP levels [173, 234, 275], and TWIST is regulated at least in part by cAMP [277]. However, in BEAS-2B cells treated with salmeterol and CSE together, there was a significant increase in TWIST signalling compared to controls. Since TWIST upregulation is also associated with EMT and depletion of E-cadherin in epithelial cells [277,

278], thisthis would suggest that salmeterol may not be a good treatment for current smokers, as cigarette smoke promoted TWIST signalling when combined with salmeterol. This study was, as noted in previous chapters, limited by the small sample sizes, which restricts the conclusions which can be drawn from the available data.

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

6.2.4. Conclusions on the effects of salmeterol

Within the limitations of this study, the only firm conclusion which could be drawn from the data was that BEAS-2B cells do not accurately represent healthy primary bronchial epithelial cells with regards to their responses to the long acting β-agonist salmeterol. The differences appeared most pronounced at the mRNA level, suggesting that if using the BEAS-2B cells line as a model protein level examination of effects is recommended over mRNA changes.

Salmeterol, despite activating cAMP and thereby potentially activating TWIST signalling, appeared to be pro-epithelial in unstimulated cells, but otherwise had no effect on EMT-related changes..

6.3. Tiotropium bromide monohydrate

6.3.1. Introduction

Tiotropium bromide (tiotropium) is a quaternary ammonium compound which acts as a long-acting muscarinic antagonist (LAMA) commonly used as a dry powder inhalation for treating asthma and COPD. It is functionally selective for M_3 receptors due to a slow dissociation rate compared to the rate of dissociation from M_2 receptors [279, 280]. Dysregulation of muscarinic receptors in airway smooth muscle has been linked to airway hyperresponsiveness in asthma and application of muscarinic antagonists such as tiotropium reduces bronchomotor tone and promotes airway widening in COPD; it is for this reason that tiotropium is applied as a bronchodilator [287]. In vitro, tiotropium has been shown to affect airway smooth muscle cell migration, which may contribute to its bronchodilatory effects in diseases with airway remodelling such as COPD [282].

Treatment with tiotropium decreases the number of exacerbations experienced by people with COPD, increases the time before first exacerbation and improves forced expiratory volume in one second (FEV_1) and quality of life in patients over four years [283, 284]. In terms of improvement inin exacerbation rates, tiotropium is more effective than both indactarol and glycopyrronium [285, 286] and despite better outcomes in the first four hours of treatment with glycopyrronium compared to tiotropium, both drugs give similar results at 28 days [285]. Additionally the SPARK trial, based on relative risk assessment, showed that tiotropium is more cost effective than glycopyyronium in the UK, Spain, Canada, and Sweden [287], making it a preferable treatment option for COPD, although there is minor evidence that it may have a mild effect on mucous clearance [288]

While the precise action of tiotropium in COPD remains unclear, a number of potential avenues of action have been demonstrated. Cigarette smoke iss known to trigger increased expression of M_3 muscarinic receptors in airway epithelial cells, in addition to increasing expression of acetylcholine which then triggers (studied via application of the agonist carbachol) an inflammatory response via TGF-β and IL-8 [282]. This inflammatory response is blocked by tiotropium. Carbachol has been demonstrated to induce EMT in alveolar epithelial cells via the Smad/ERK pathways [289], and TGF-β is known to induce EMT in airway epithelial cells [165, 207, 257]. Both TGF-β and carbcahol-induced EMT can be blocked by the application of an M_3 antagonist, such as tiotropium [289] which suggests that tiotropium may not only be acting on the inflammatory aspect of COPD but may also contribute to blocking pathological changes in the epithelium. This effect on the epithelial layer is of special interest to this thesis, and the focus of this study.

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COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

6.3.2. Results

6.3.2.1. Effects of tiotropium on EMT markers in primary and BEAS-2B cells

6.3.2.1.1. Epithelial markers

Tiotropium did not affect expression of E-cadherin at the protein level in primary or BEAS-2B cells, although in both cell types it reduced expression in samples which initially expressed high levels (Figure 6.3-1). At the mRNA level, BEAS-2B cells were unaffected by tiotropium, except for a single expressing sample which initially expressed high levels of E-cadherin and decreased expression to become consistent with the other samples following treatment, while all primary cell groups werewere unaffected.

In both primary and BEAS-2B cells, cytokeratin expression was unaffected by tiotropium exposure (Figure 6.3-2). Likewise, primary cells treated with tiotropium exhibited no change in tight junction protein (TJP1) mRNA expression (Figure 6.3-3). Overall, epithelial markers were unaffected by exposure to tiotropium.

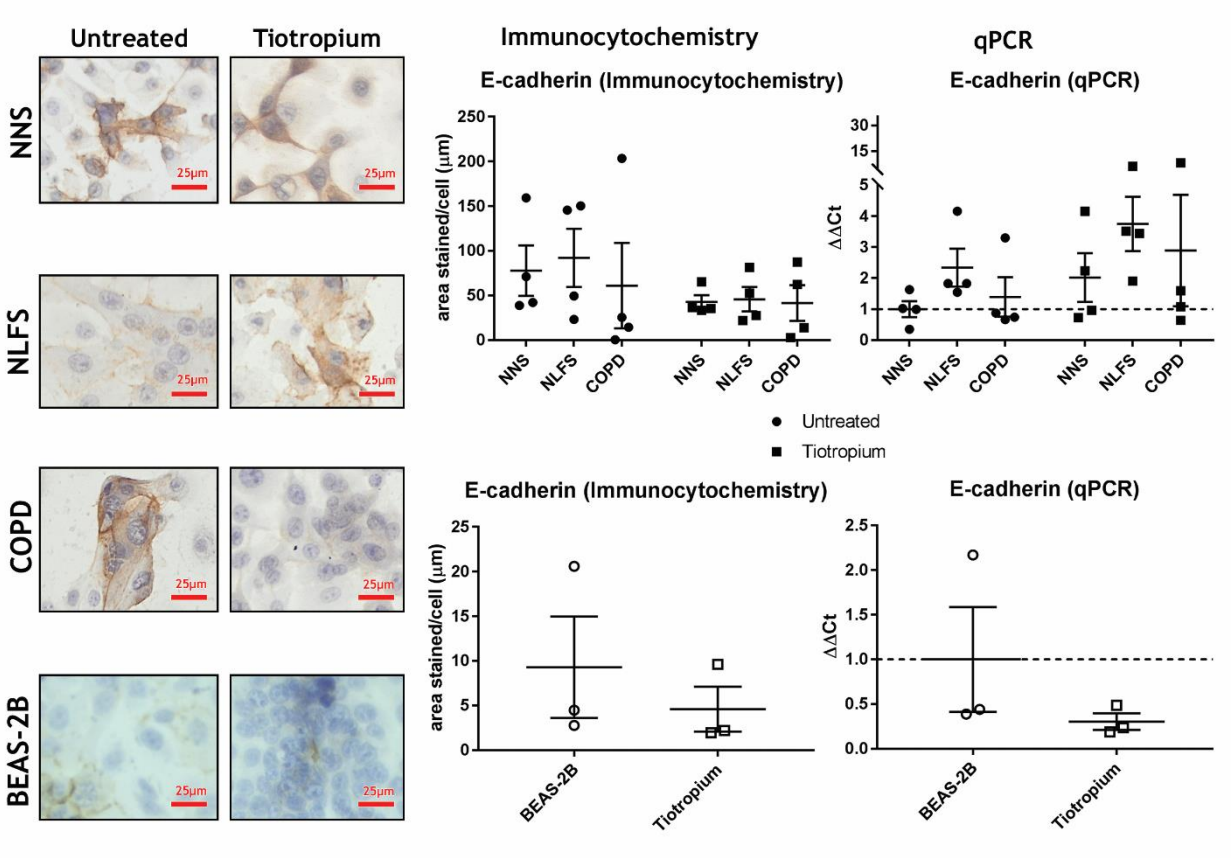

Figure 6.3-1: Expression of E-cadherin in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD)people with airflow limitation (COPD) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to tiotropium for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells. ΔΔCT shows the fold-change in expression, with '1' being no change from baseline or the control.*

Key: *Filled shapes are pHBECs, open shapes are BEAS-2B cells.*

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

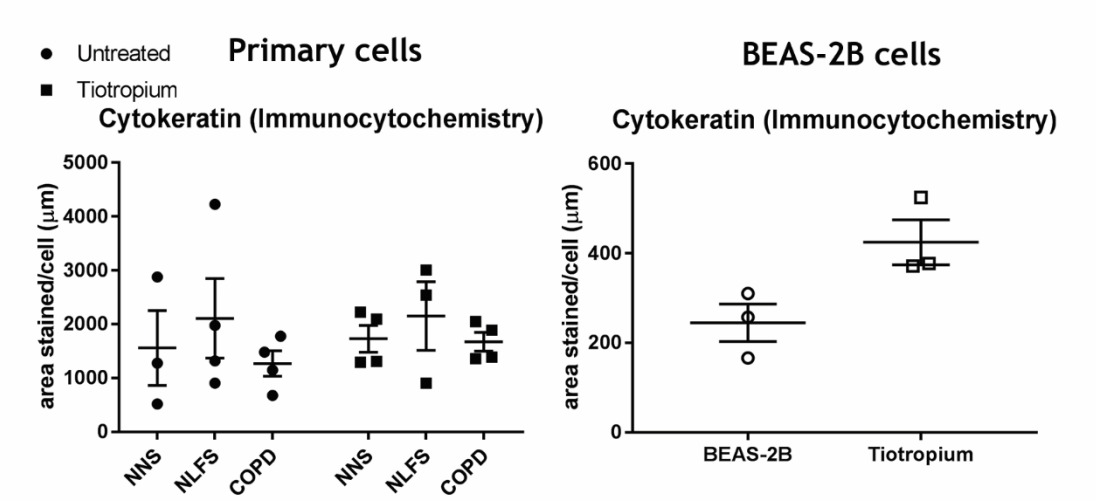

Figure 6.3-2: Expression of cytokeratin in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by immunocytochemistry with and without exposure to tiotropium for 24 hours. *Data are represented as mean with SEM. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

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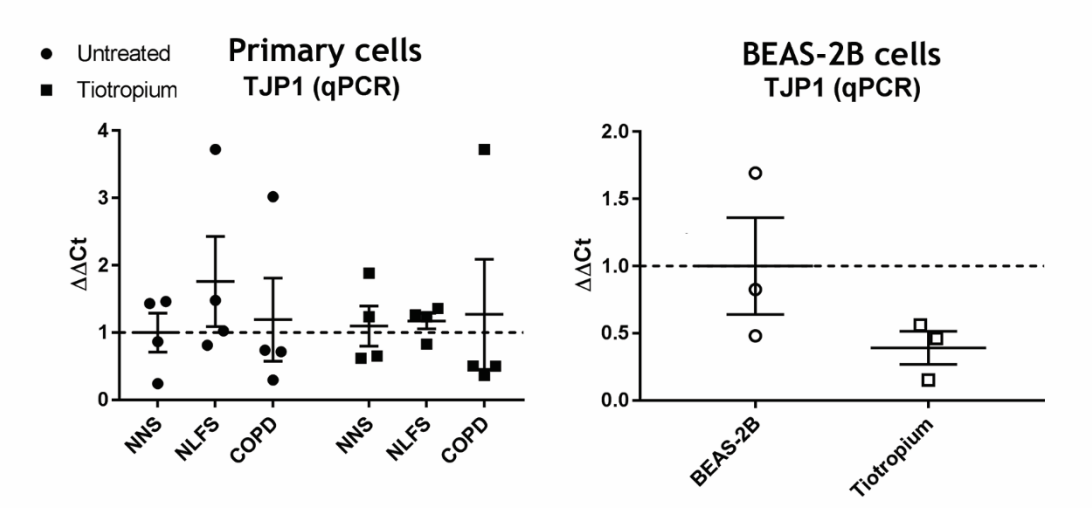

Figure 6.3-3: Expression of tight junction protein-1 (TJP1) in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by qPCR with and without exposure to tiotropium for 24 hours. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

6.3.2.1.2. Mesenchymal markers

Treatment with tiotropium reducede variability between samplessamples in cells from smokers with normal lung function and people with airflow obstruction (Figure 6.3-4). At the mRNA level the trend in primary cells was the same, with primaryprimary cells in all three groups being generally unaffected, and ini BEAS-2B cells tiotropium had no effect on N-cadherin expression at either the protein or mRNA level.

Tiotropium did not affect protein level expression of vimentin in either primary or BEAS-2B cells, nor did it affect mRNA expression in BEAS-2B cells (Figure 6.3-5). However, at the mRNA level in pHBECs tiotropium increased vimentin expression 15-fold in non-smokers' cells, and if the untreated samples from all three groups were combined into a single 'baseline' group then the increase in non-smokers' cells following treatment reached statistical significance (unpaired t-test $p<0.0001$).

S100A4 expression increasedappeared to increase in aa subset of non-smokers' samples following tiotropium exposure, however these changes could not be confirmed statistically and most primary cells and the BEAS-2B cells' expression of S100A4 were unaffected by the drug (Figure 6.3-6).

Overall, tiotropium hadhave limited effect on mesenchymal markers in primary cells from non-smokers,with other primary cells and BEAS-2B cells being unaffected.

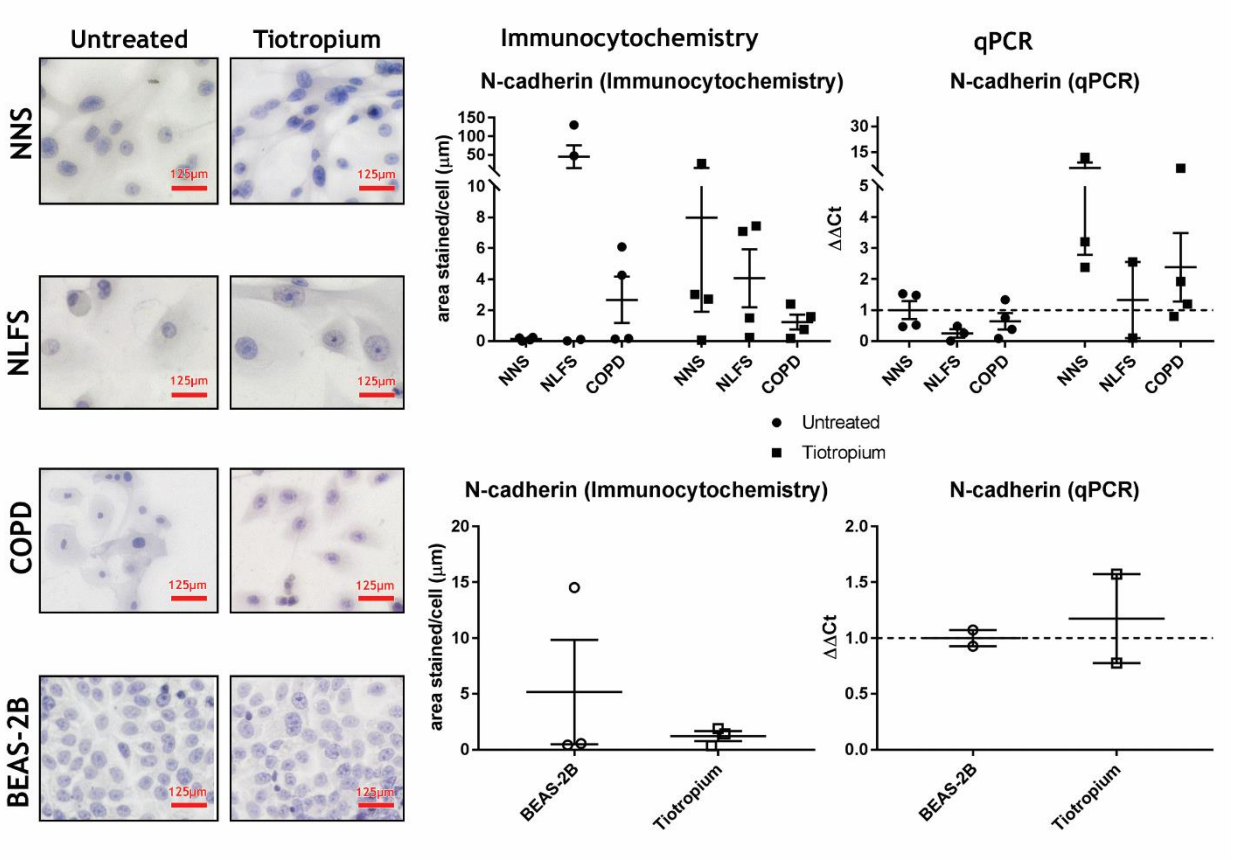

Figure 6.3-4: Expression of N-cadherin in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to tiotropium for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

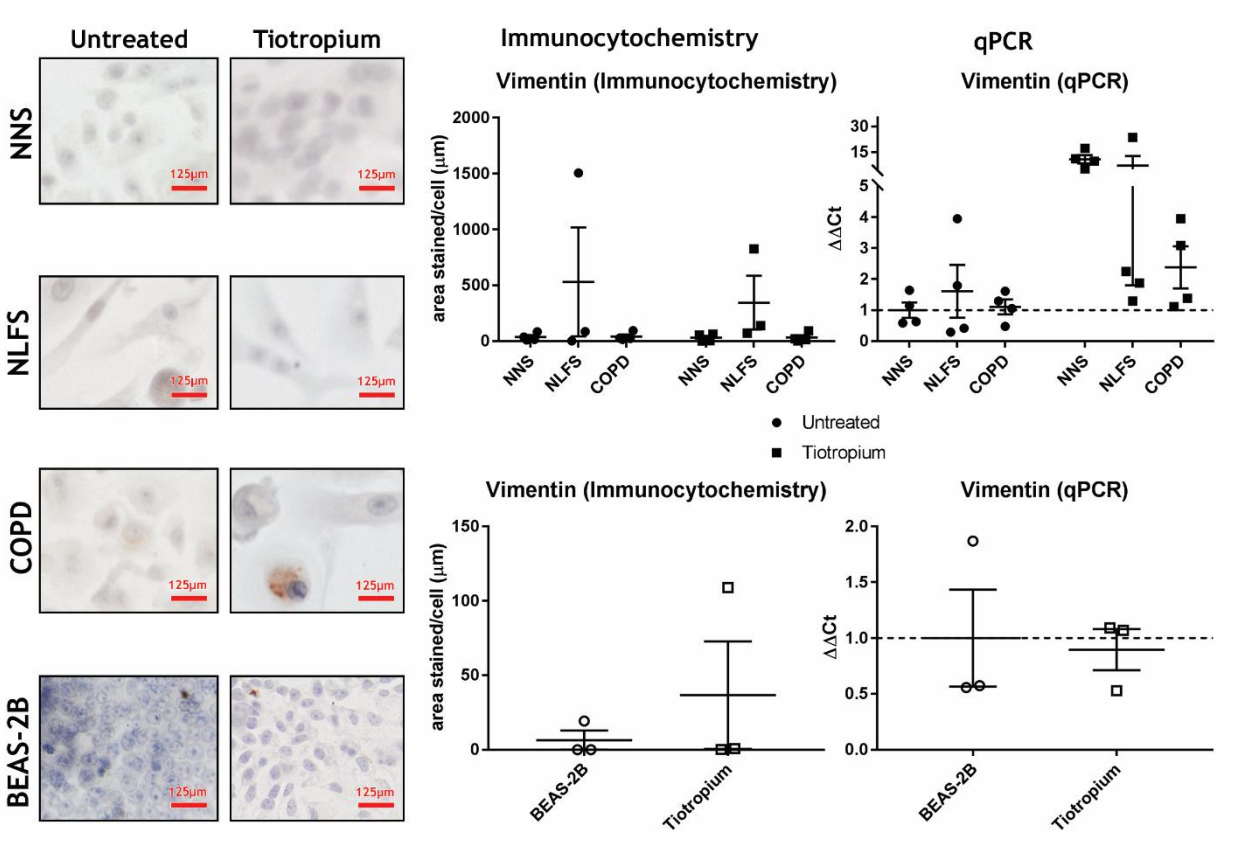

Figure 6.3-5: Expression of vimentin in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to tiotropium for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

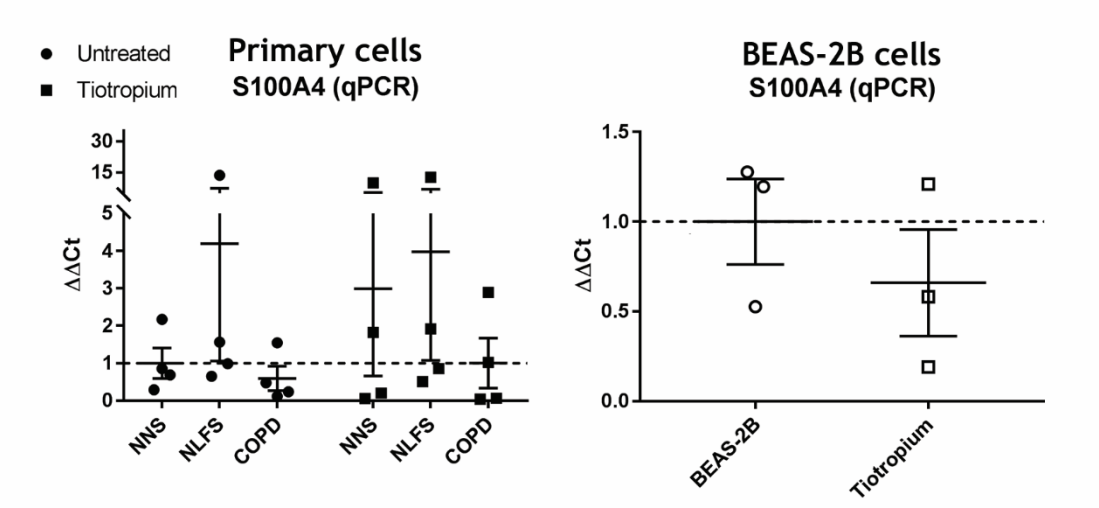

Figure 6.3-6: Expression of S100A4 in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by qPCR with and without exposure to tiotropium for 24 hours. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

6.3.2.1.3. Signalling molecules

Exposure to tiotropium had no effect on any of the three primary cell groups' expression of TWIST at the mRNA level, nor did it affect expression in BEAS-2B cells (Figure 6.3-7). SimilarlySimilarly, in both primary cells and BEAS-2B cells, tiotropium hadhave no effect on Smad6 expression (Figure 6.3-8). Overall, tiotropium hadhave no effect on either TWIST or Smad6 signalling.

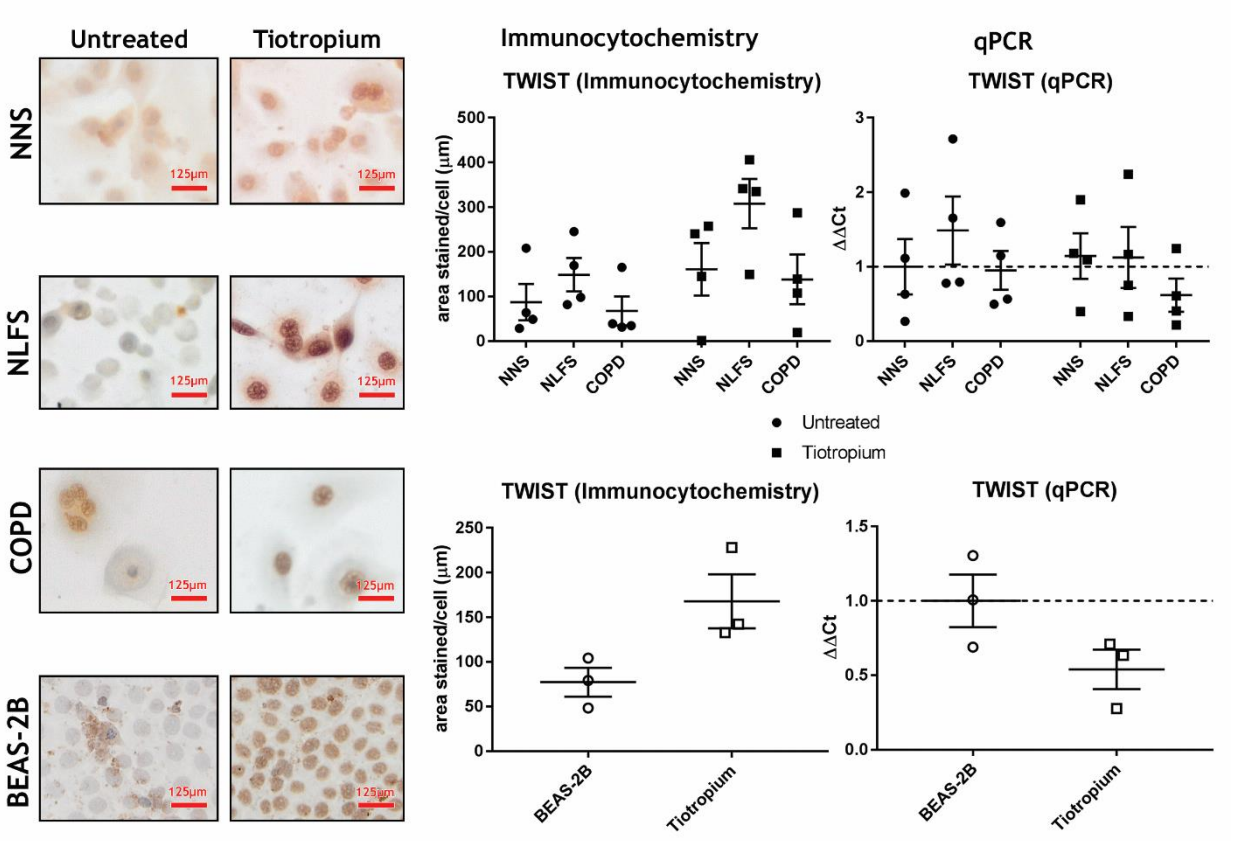

Figure 6.3-7: Expression of TWIST in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to tiotropium for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

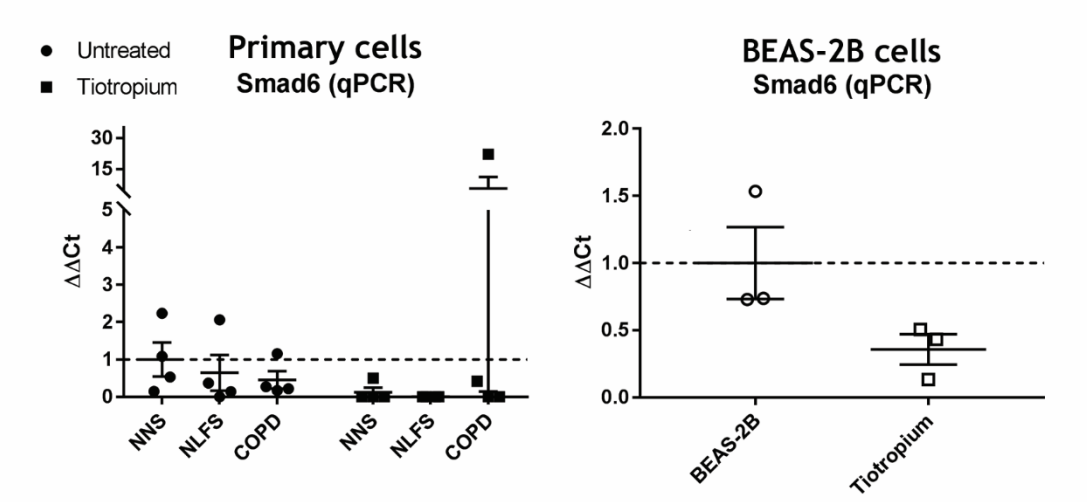

Figure 6.3-8: Expression of Smad6 in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by qPCR with and without exposure to tiotropium for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

6.3.2.1.4. Extracellular matrix remodelling

Application of tiotropium hadhad no effect on expression of MMP2 in either primary cells or BEAS-2B cells (Figure 6.3-9).

Collagen 1-α expression, both at the protein and mRNA level was unaffected in BEAS-2B cells, as well as in the majority of primary cells taken from people with airflowairflow limitation, although a single sample exhibited a sharp increase in expression in response to the drug. In cells taken from non-smokers and smokers with normal lung function, tiotropium had no effect on either collagen 1-α or MMP2.

Overall, tiotropium possibly ahad no effect on extracellular remodelling proteins in either primary orin BEAS-2B cells.

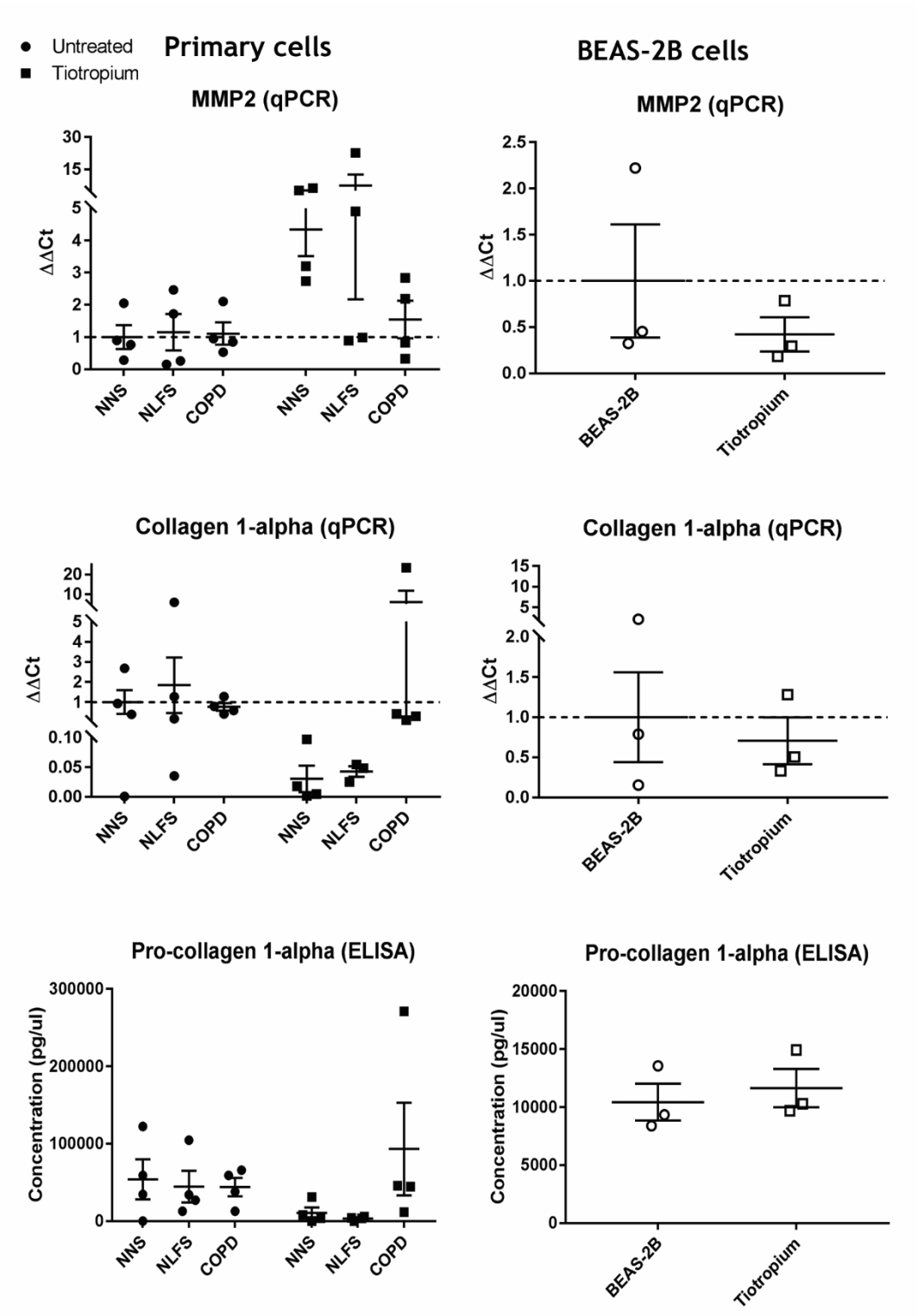

Figure 6.3-9: Expression of MMP2 and collagen 1-α in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by ELISA of cell culture supernatant and qPCR with and without exposure to tiotropium for 24 hours. *Data are represented as mean with SEM.* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

Useful abbreviations

NNS *– non-smokers*
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LABA/LAMA – *long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

6.3.2.2. TGF-β and CSE-induced EMT

6.3.2.2.1. Epithelial markers

Tiotropium had no effect on E-cadherin expression in either primary cells or BEAS-2B cells treated with transforming growth factor-β1 (TGF-β) or cigarette smoke extract (CSE) (Figure 6.3-10).

Tiotropium had no effect on cytokeratin expression in primary cells treated with either TGF-β or CSE, nor did it affect expression in BEAS-2B cells treated with TGF-β (Figure 6.3-11). However, BEAS-2B cells treated with TGF-β and tiotropium exhibited a 1.7 times increase (one-way ANOVA p = 0.0127) in cytokeratin expression following exposure compared to cells treated with only TGF-β. raise the expression to nearExpression of tight junction protein-1 (TJP1) in cells treated with TGF-β and tiotropium exhibited no change in expression in either primary cells or BEAS-2B cells (Figure 6.3-12).

Overall, the combination of TGF-β and tiotropium did not affect epithelial marker expression in the primary cells, except in primary cells treated with CSE.

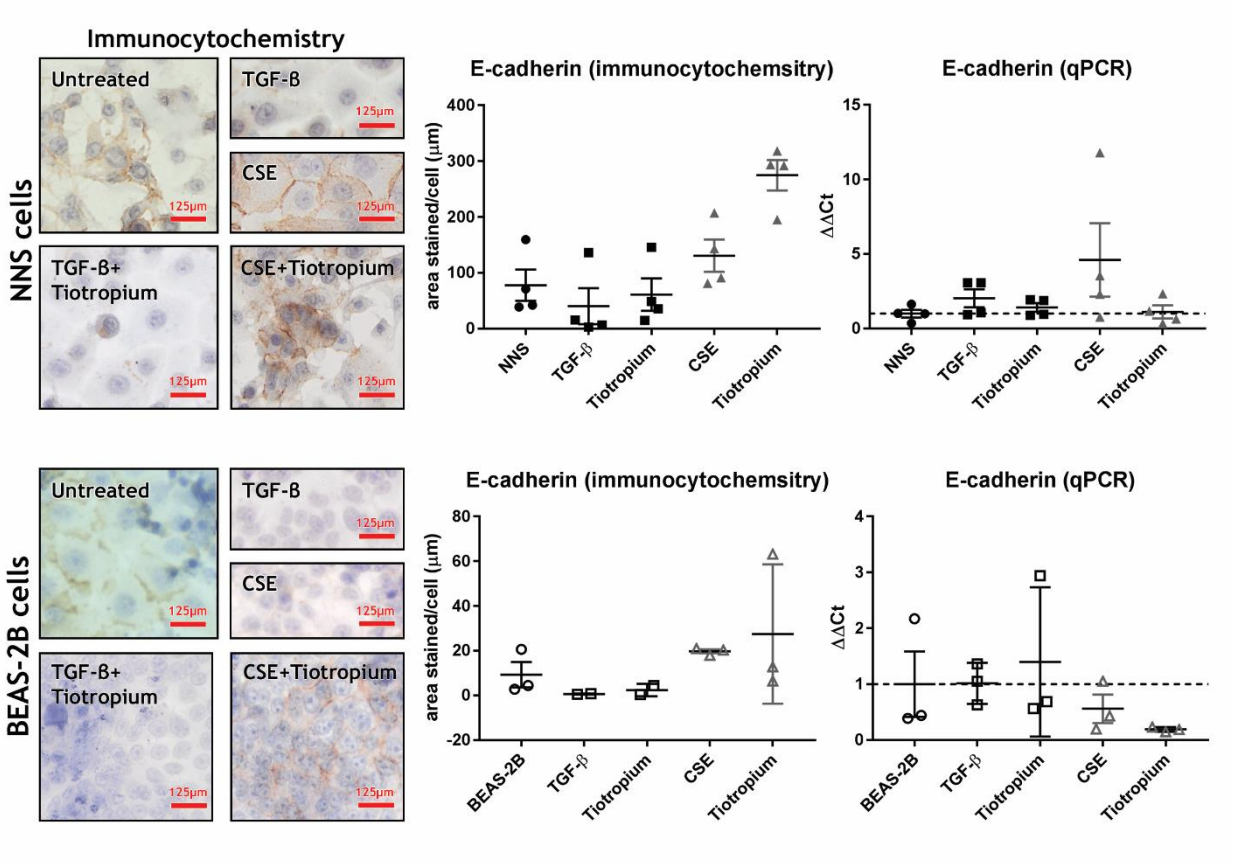

Figure 6.3-10: Expression of E-cadherin in non-smokers (NNS) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with tiotropium for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

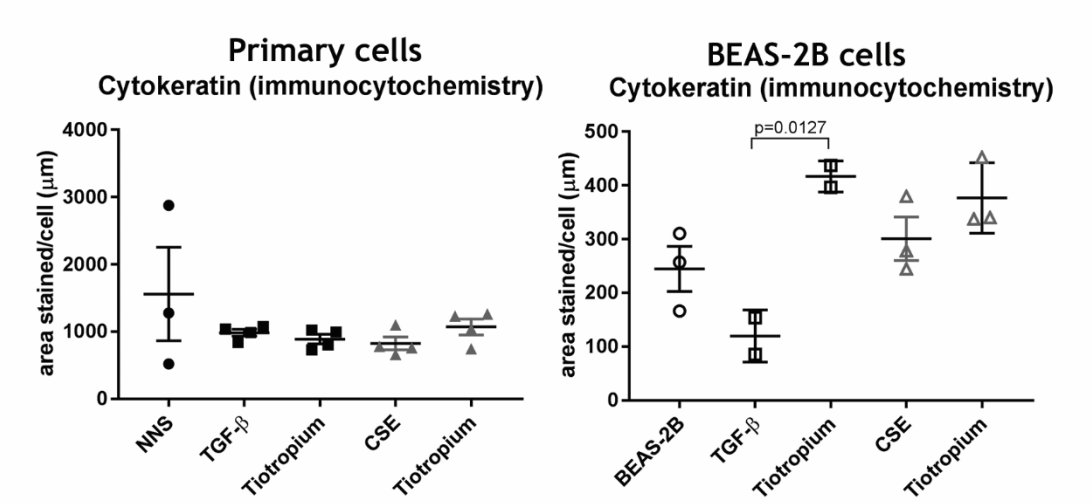

Figure 6.3-11: Expression of cytokeratin in non-smokers (NNS) and immortalised BEAS-2B cells as measured by immunocytochemistry with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with tiotropium for 24 hours. *Data are represented as mean with SEM. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).*

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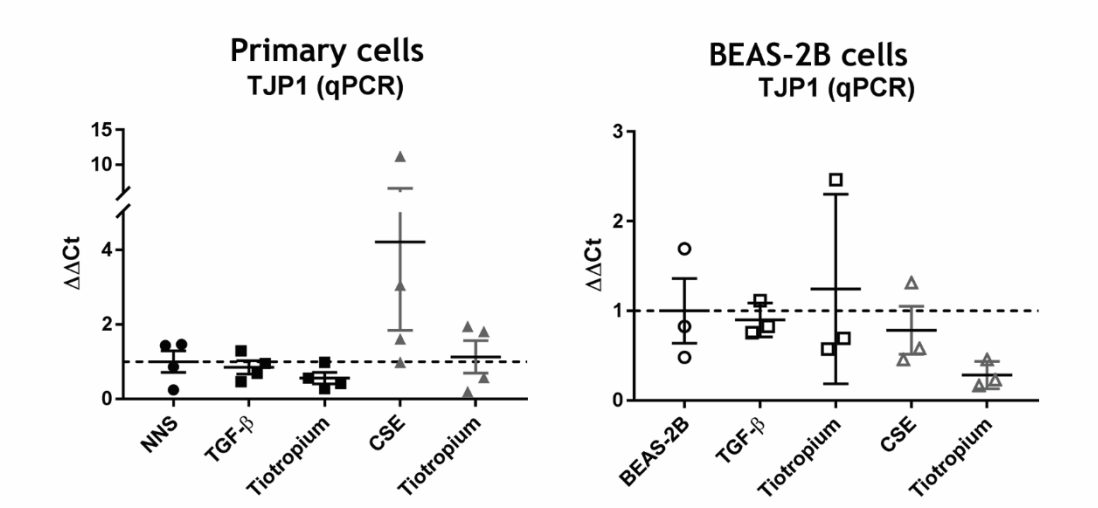

Figure 6.3-12: Expression of tight junction protein-1 (TJP1) in non-smokers (NNS) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with tiotropium for 24 hours. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

6.3.2.2.2. Mesenchymal markers

In primary and BEAS-2B cells exposed to either transforming growth factor-β1 (TGF-β) or cigarette smoke extract (CSE) tiotropium appeared to have no effect on the expression of N-cadherin (Figure 6.3-13). While the expression of N-cadherin in primary cells treated with tiotropium in combination with TGF-β or CSE appeared statistically to return to baseline expression at the protein (TGF-β treated cells) and mRNA (CSE treated cells) level, it is clear thatthe this change is likelyprobably due to changes in variabilityvariation between samples rather than a true effect.

Expression of vimentin in both primary and BEAS-2B cells exposed to TGF-β was not affected by the application of tiotropium (Figure 6.3-14). At the protein and mRNA level, cells exposed to CSE and tiotropium likewise exhibited no change in vimentin expression.S100A4 expression was unaffected by tiotropium in either primary or BEAS-2B cells exposed to TGF-β or CSE (Figure 6.3-15).

Overall, tiotropium had no effect on the expression of mesenchymal markers in either primary or BEAS-2B cells exposed toto TGF-β or CSE.

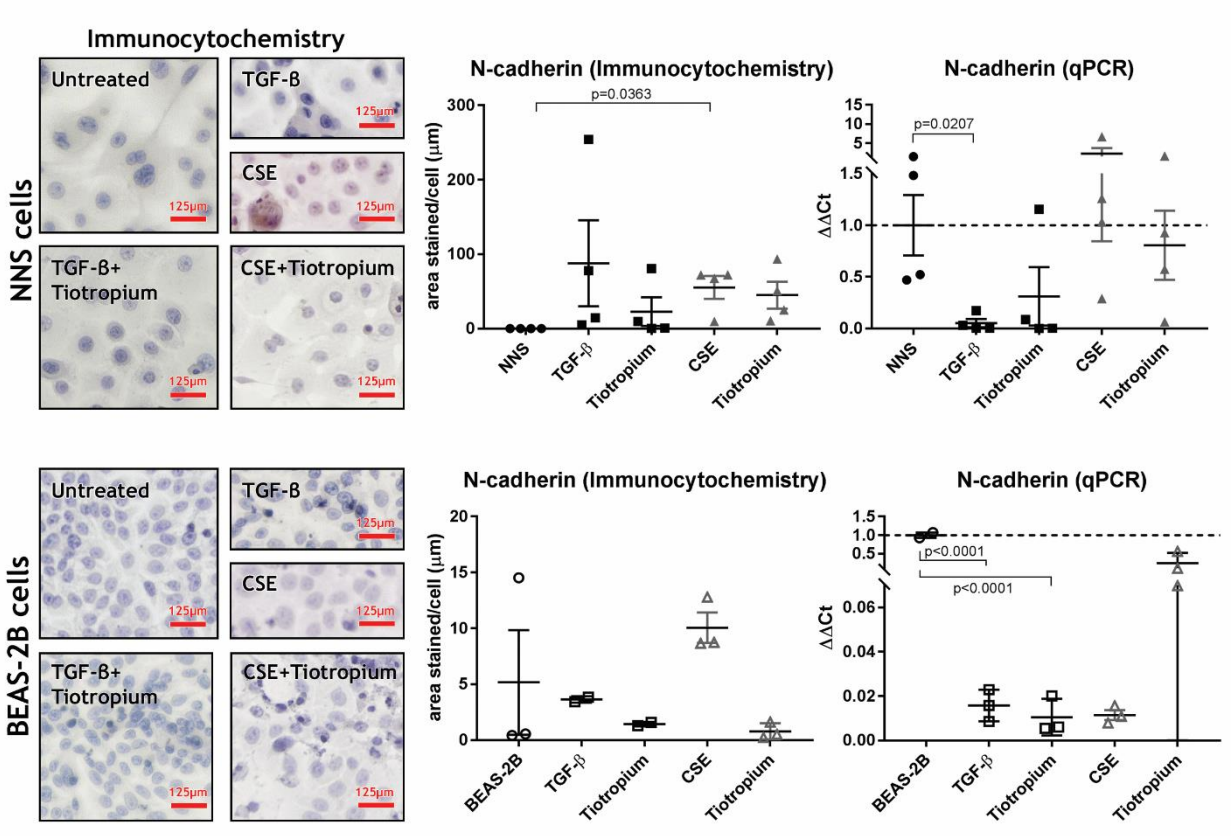

Figure 6.3-13: Expression of N-cadherin in non-smokers (NNS) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with tiotropium for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

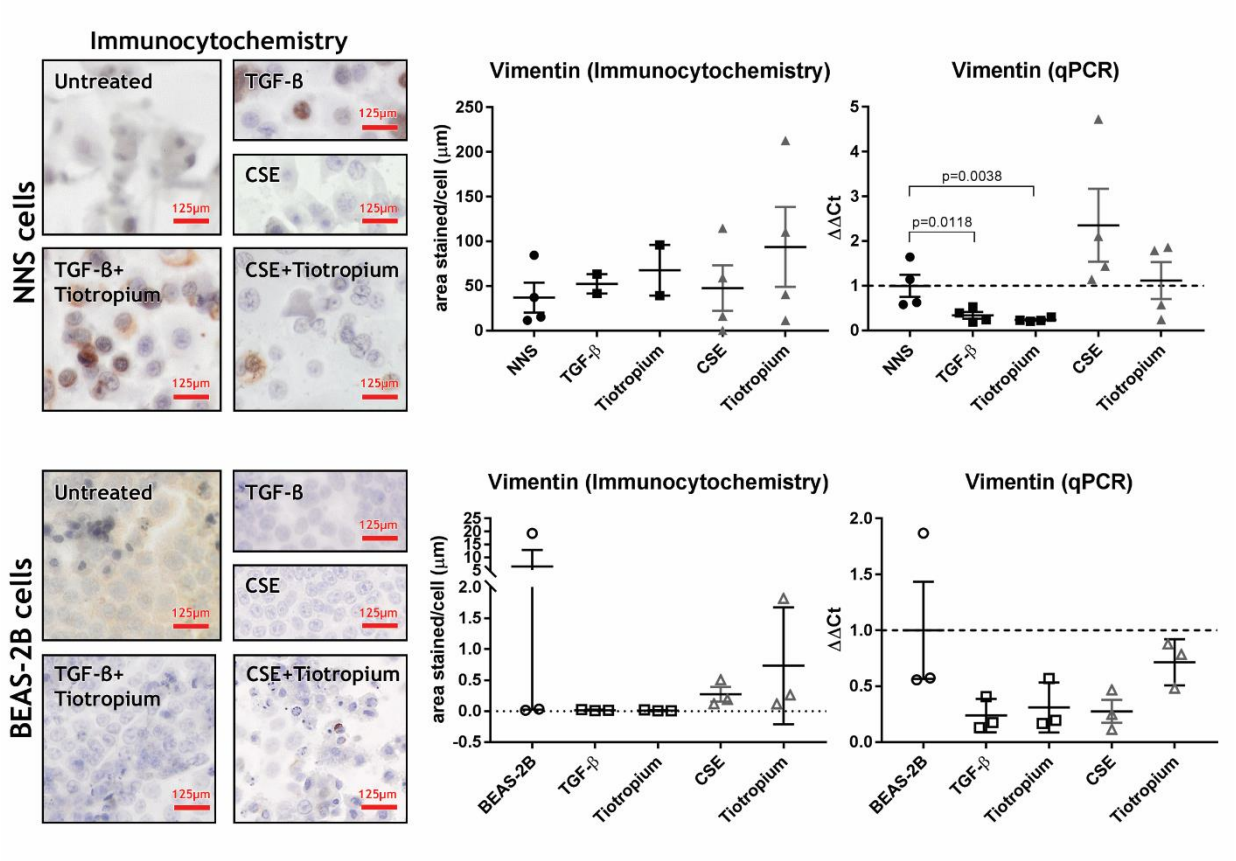

Figure 6.3-14: Expression of vimentin in non-smokers (NNS) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with tiotropium for 24 hours. Exposure to TGF-β and tiotropium caused no change in vimentin expression as compared to TGF-β alone in either primary or BEAS-2B cells. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

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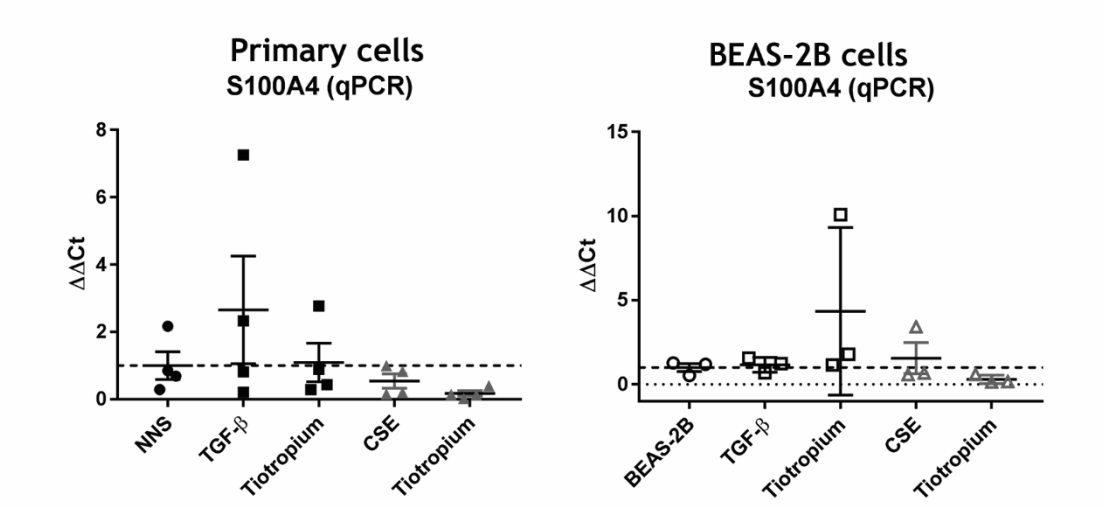

Figure 6.3-15: Expression of S100A4 in non-smokers (NNS) and immortalised BEAS-2B cells as measured by qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with tiotropium for 24 hours. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

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TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

6.3.2.2.3. Signalling molecules

The combination of transforming growth factor-β1 (TGF-β) and tiotropium had no effect on TWIST expression in either primary or BEAS-2B cells (Figure 6.3-16). Likewise, the combination of cigarette smoke extract (CSE) and tiotropium had no effect on TWIST expression in either primary or BEAS-2B cells. Tiotropium had no effect on Smad6 expression in primary or BEAS-2B cells exposed to TGF-β or CSE (Figure 6.3-17).

Overall, neither primary nor BEAS-2B cells exhibited any response in the TWIST or Smad6 signalling molecules following treatment with tiotropium and exposure to either TGF-β or CSE.

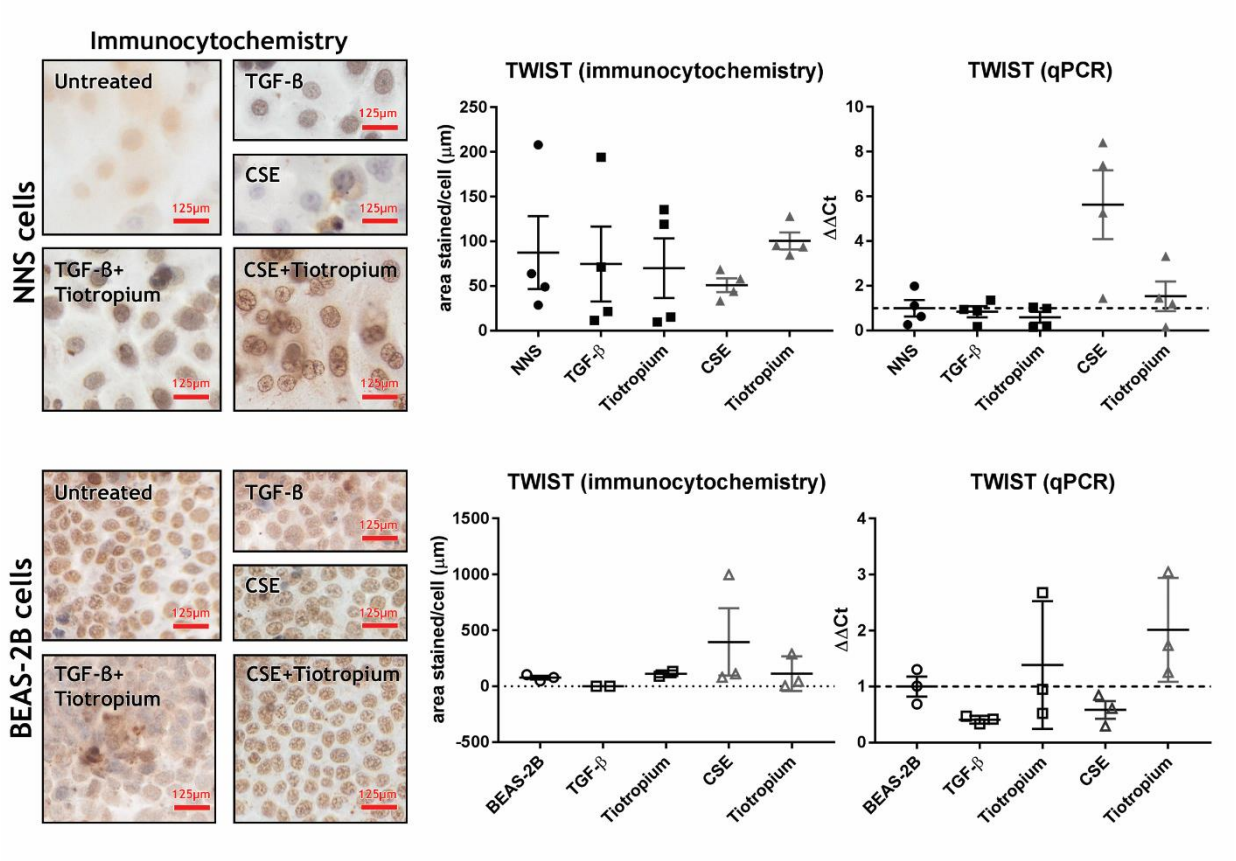

Figure 6.3-16: Expression of TWIST in non-smokers (NNS) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with tiotropium for 24 hours. In primary cells treated with TGF-β tiotropium had no effect on TWIST expression at either the protein or mRNA level. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

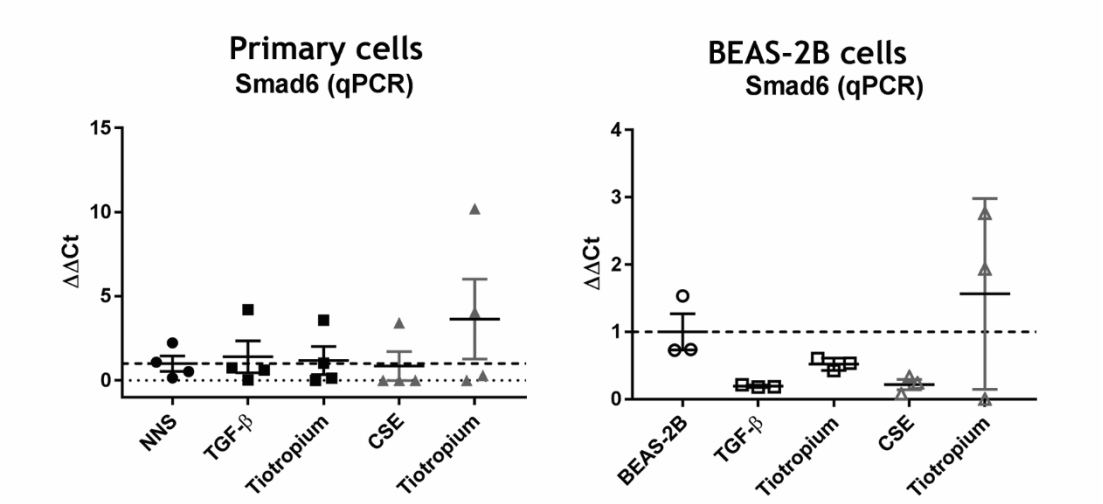

Figure 6.3-17: Expression of Smad6 in non-smokers (NNS) and immortalised BEAS-2B cells as measured by qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with tiotropium for 24 hours. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

6.3.2.2.4. Extracellular matrix remodelling

Both primary and BEAS-2B cells exposed to transforming growth factor-β1 (TGF-β) and tiotropium exhibited no change in the expression of MMP2 compared to cells exposed to TGF-β alone, nornor did tiotropium affect MMP2 expression in cells treated the cigarette smoke extract (CSE) (Figure 6.3-18). Tiotropium hadhad no effect on the expression of collagen 1-α and pro-collagen 1-α in primary and BEAS-2B cells exposed to TGF-β or CSE. ,although di dnotOverall, tiotropium appeared to have no effect on extracellular matrix remodelling proteins.

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

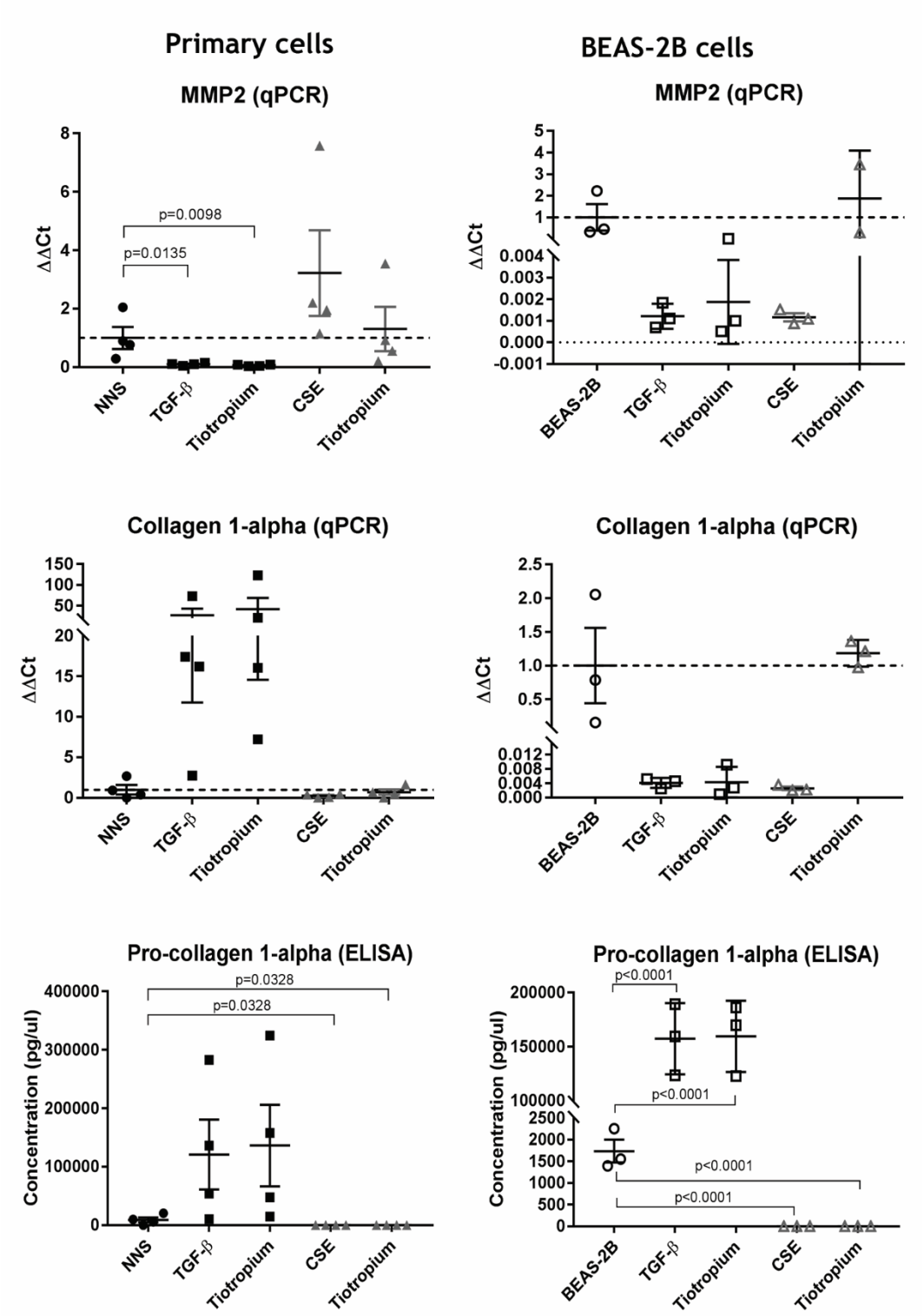

Figure 6.3-18: Expression of MMP2 and collagen 1-α in non-smokers (NNS) and immortalised BEAS-2B cells as measured by ELISA of cell culture supernatant and qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with tiotropium for 24 hours. *Data are represented as mean with SEM.* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

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pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

6.3.3. Discussion of the effects of tiotropium

In this study, primary bronchial epithelial cells taken from non-smokers appeared to demonstrate similar reactions to cells taken from people with airflow limitation when treated with tiotropium. Specifically, there was no change in epithelial or mesenchymal marker expression in response to tiotropium. Tiotropium had no effect on expression of fibrotic extracellular matrix protein collagen 1-α or MMP2. Tiotropium overall appeared to have no effect on EMT in untreated cells, nor in primary or BEAS-2B cells treated with either transforming growth factor-β1 (TGF-β) or cigarette smoke extract (CSE). The only exception to this is cells from non-smokers, which demonstrated an increase in vimentinin response to tiotropium, suggesting limited pro-EMT activity.

Despite the fact that tiotropium is known to inhibit TGF-β-induced EMT [289] it appeared to have no effect on Smad6 levels in this study. The reason for this is unclear, however it is possible that although carbachol induces EMT via the Smad pathway, tiotropium acts via a different mechanism to reduce EMT. There may also be variation between the action of tiotropium in alveolar epithelial cells, used in Yang and colleagues' study [289], and epithelial cells taken from the airways, utilised in this study. It is likely that the lack of effect seen in this study is due to the failure to induce an active EMT phenotype in the cells, resulting in tiotropoum having no apparent effect.

6.3.4. Conclusions on the effect of tiotropium

BEAS-2B cells appeared to be a reasonably accurate representation of primary bronchial epithelial cells' responsescells to the long acting muscarinic antagonist tiotropium, however, there was a significant difference in the cells' responses to tiotropium when observing the mesenchymal marker vimentin.. Tiotropium may be detreimental to healthy non-smokers, inducing a mildly mesenchymal phenotype, however since tiotropium is used to treat established disease this result, while interesting, is not of clinical relevance.. Tiotropium appeared to have no effect on induced EMT in primary cells, although this was likely due to failure to induce full EMT via application of transforming growth factor-β1 or cigarette smoke extract.

6.4. Fluticasone propionate

6.4.1. Introduction

Fluticasone is a glucocorticoid used to treat COPD [290]. It is known that COPD is generally resistant to inhaled corticosteroid (ICS) treatment, however co-treatment with a long-acting β-agonist (LABA) has been shown to rescue the corticosteroid resistant phenotype induced in bronchial epithelial cells by inflammatory mediators [291]. As an additional note, the phosphodiesterase-4 inhibitor roflumilast reverses corticosteroid resistance in neutrophils in COPD [56], once again emphasising that this is a complex disease with a number of inter-linked aspects underlying its pathology.

The global initiative for chronic obstructive lung disease (GOLD) recommendations for the management of stable COPD do not recommend ICS monotherapy, although they do not condemn it [208]. This may be due to the fact that corticosteroid treatment increases the risk of lower respiratory tract infections, septicaemia [298] and both contracting pneumonia [299] and experiencing recurring pneumonia infections [294]. However GOLD does note that dual or triple therapy in combination with one or more bronchodilators, particularly LABAs, is of benefit in moderate to severe COPD [208].

While corticosteroids are often considered to act on inflammation in COPD, targeting the immune cells, there is evidence that they can act directly on the epithelial cells [295-298]. Of particular relevance to this study is the fact that corticosteroid exposure, specifically dexamethasone, has been shown to reduce the ability of epithelial cells to repair wounds, a process which involves epithelial-mesenchymal transition [305].

6.4.2. Results

6.4.2.1. Effects of fluticasone on EMT markers in primary and BEAS-2B cells

6.4.2.1.1. Epithelial markers

Fluticasone have no effect on the expression of E-cadherin in the three primary cell groups, not in BEAS-2B cells (Figure 6.4-1).

Exposure to fluticasone also had no effect on the expression of cytokeratin in the primary or BEAS-2B cells(Figure 6.4-2) nornor did it affect tight junction protein-1 (TJP1) expression in any of the primary groups, although one individualividu in the non-smoker group did greatly increase expression following treatment (Figure 6.4-3).

Overall, fluticasone hadhave no effect on the expression of epithelial markers in either primary or immortalised cells.

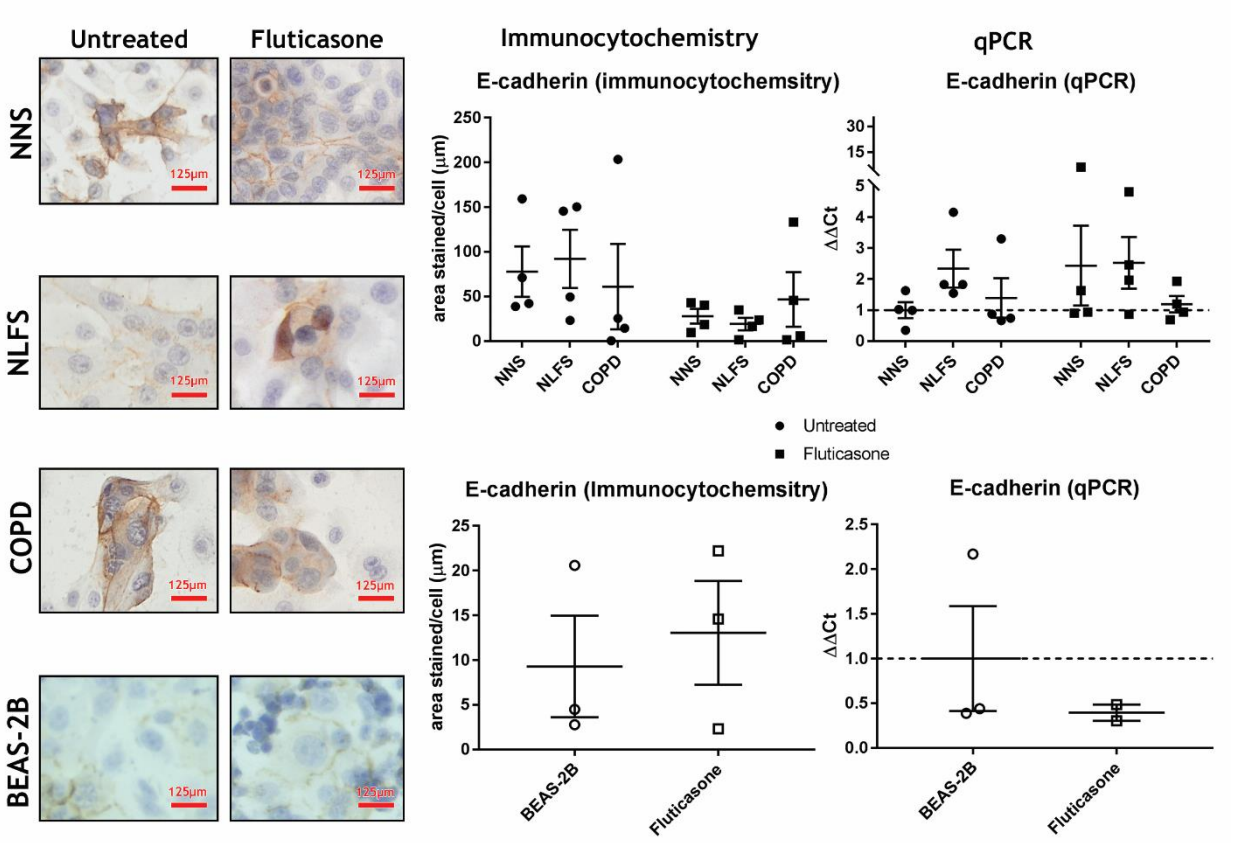

Figure 6.4-1: Expression of E-cadherin in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to fluticasone for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

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CSE *– cigarette smoke extract*

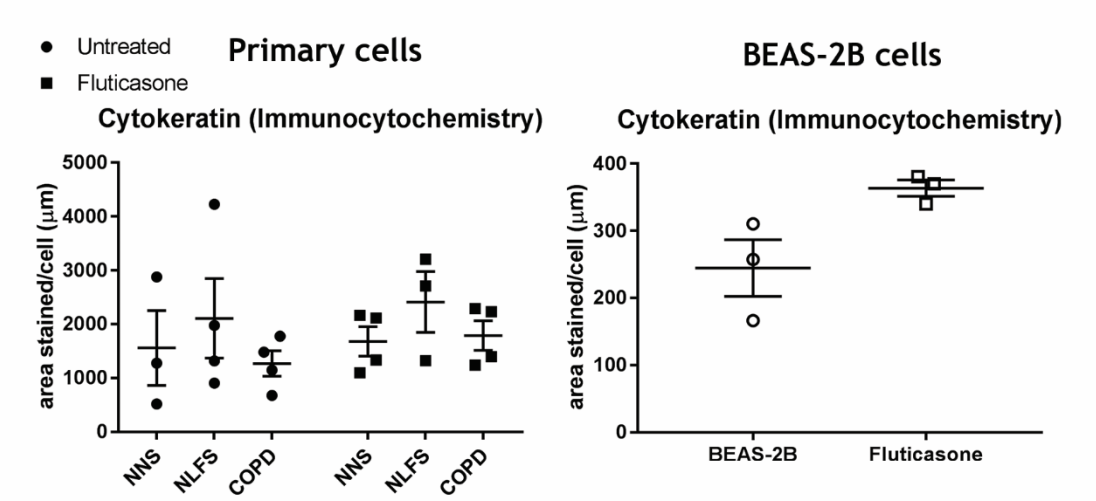

Figure 6.4-2: Expression of cytokeratin in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by immunocytochemistry with and without exposure to fluticasone for 24 hours. *Data are represented as mean with SEM. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

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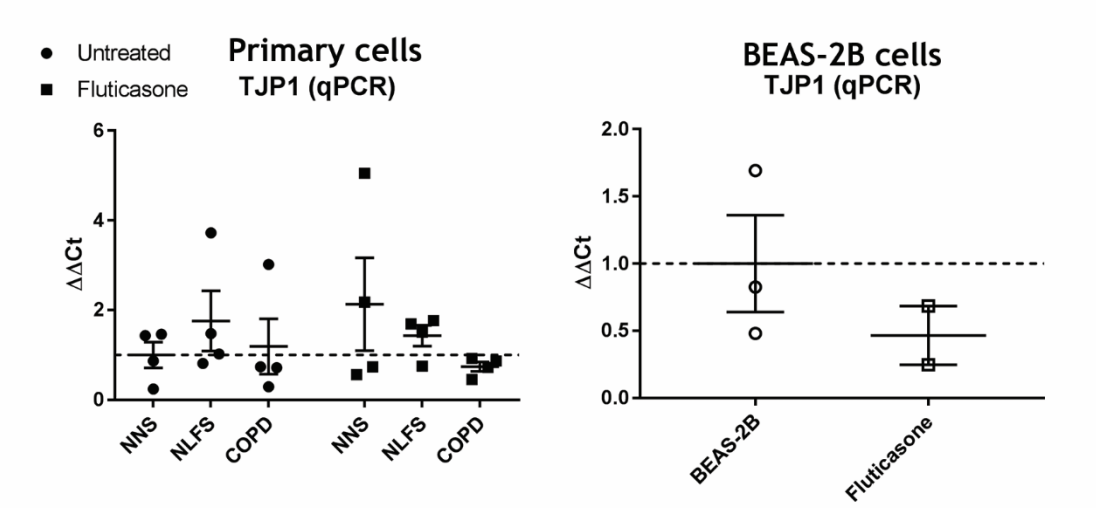

Figure 6.4-3: Expression of tight junction protein-1 (TJP1) in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by and qPCR with and without exposure to fluticasone for 24 hours. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

6.4.2.1.2. Mesenchymal markers

Primary cells taken from non-smokers and people with airflow obstruction did not show alteredexpression of N-cadherin in response to fluticasone, although cells from smokers which initially expressed higher levels of N-cadherin protein reduced expression to the same level as the other groups following treatment (Figure 6.4-4). Similarly, BEAS-2B samples expressing initially high levels of N-cadherin protein appeared to decrease expression following exposure to fluticasone. Primary and BEAS-2B cells generally exhibited no change in expression of N-cadherin mRNA in response to fluticasone, although two primary samples from non-smokers appeared to greatly increase expression in response to the drug.

Vimentin expression in response to fluticasone followed a similar pattern to N-cadherin expression, with primary cells which initially expressed high levels of vimentin protein decreasing expression, and a subset of non-smoker samples increasing mRNA level expression following exposure (Figure 6.4-5). Once again, the BEAS-2B cells' expression of vimentin protein remained unchanged after exposure. BothBoth primary and BEAS-2B cells' expression of S100A4 mRNA was unchanged following treatment with fluticasone, with the exception of a sample from a smoker with normal lung function which initially expressed high levels of S100A4 and decreased expression following treatment.

Overall, it would appear that primary cells expressing low levels of mesenchymal markers were unaffected by fluticasone, although those which expressed high levels decreased in in response to the drug. However,, however broadly speaking fluticasone did not have any significant effect on mesenchymal markers in either primary or immortalised cells.

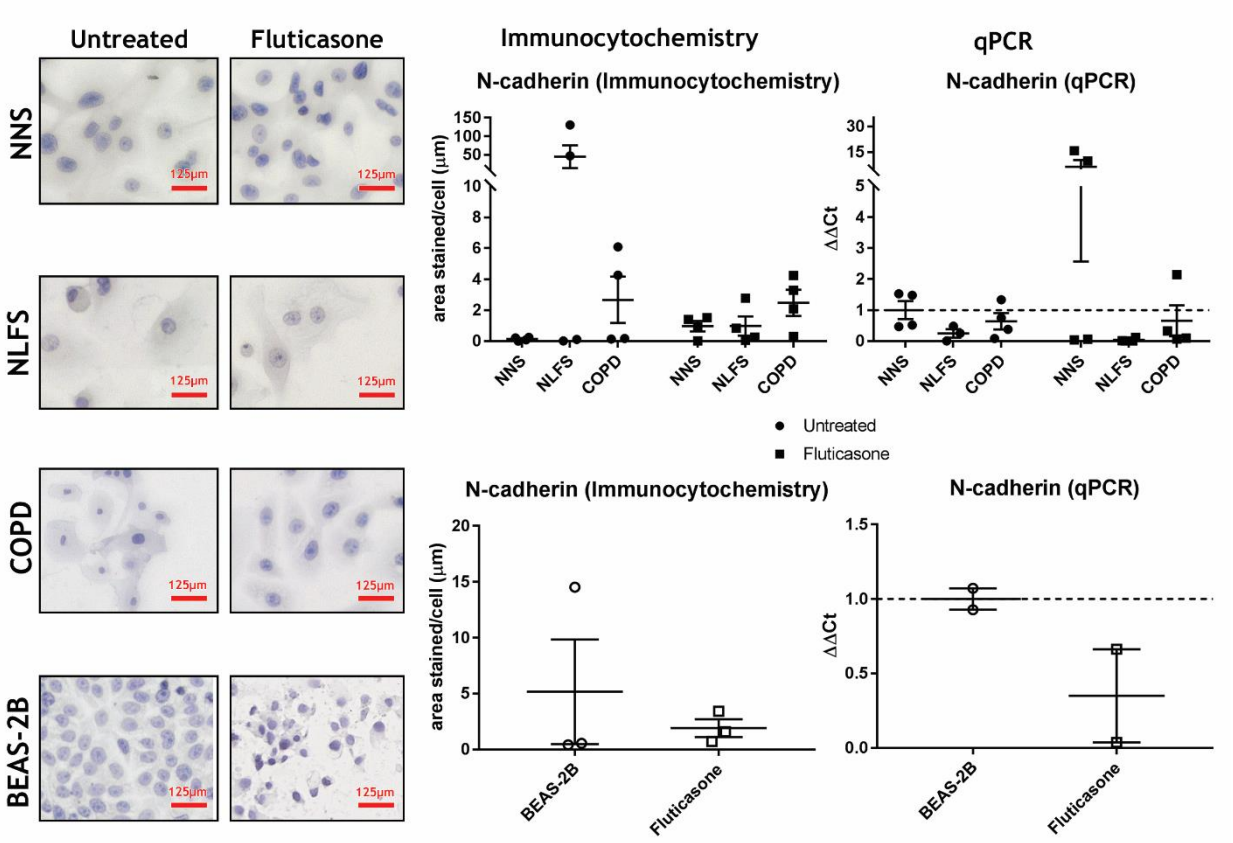

Figure 6.4-4: Expression of N-cadherin in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to fluticasone for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

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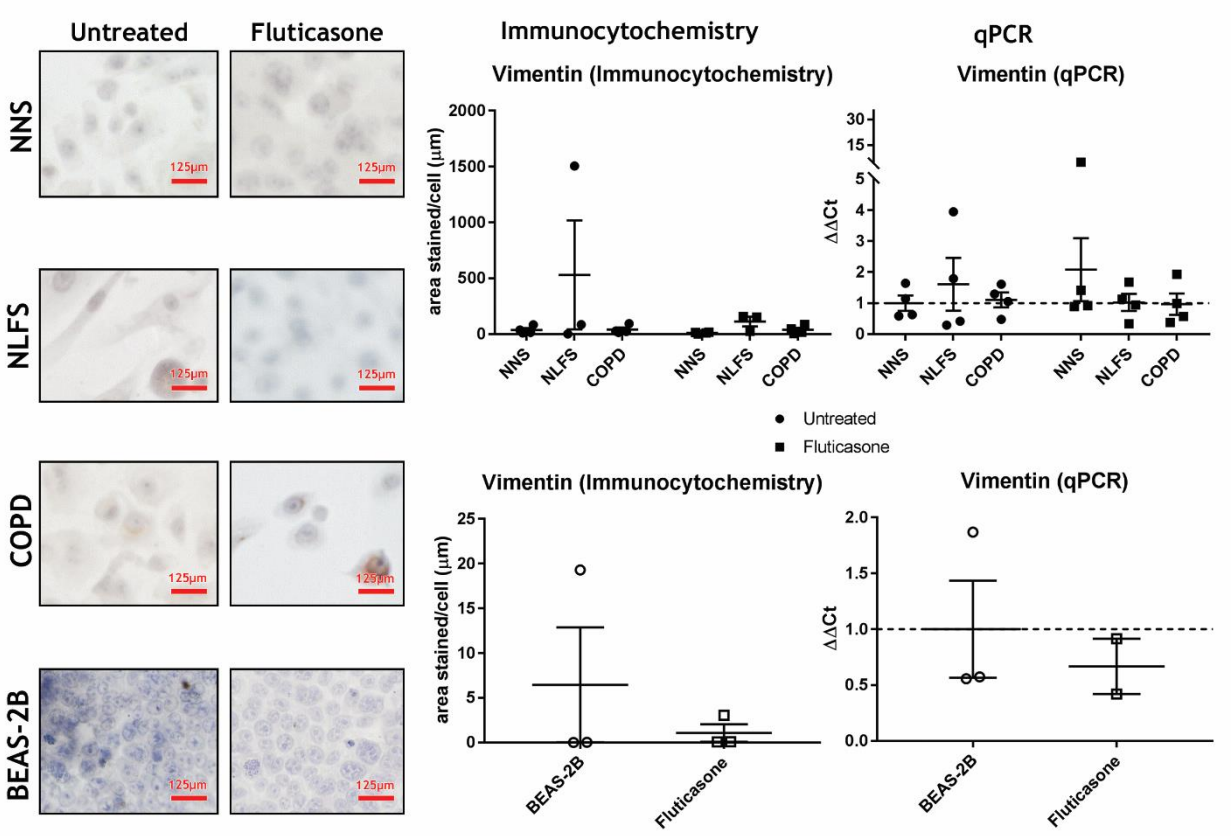

Figure 6.4-5: Expression of vimentin in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to fluticasone for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

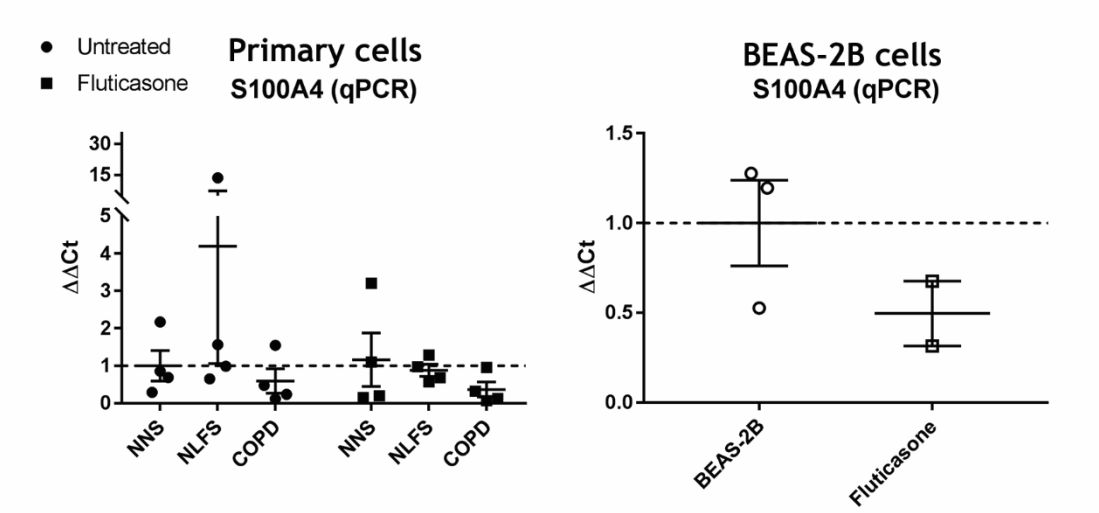

Figure 6.4-6: Expression of S100A4 in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by qPCR with and without exposure to fluticasone for 24 hours. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

6.4.2.1.3. Signalling molecules

Both primary cells and BEAS-2B cells demonstrated no change in the expression of TWIST at either the protein or mRNA level (Figure 6.4-7) following treatment with fluticasone. Smad6 expression in primary cells taken from non-smokers and people with airflow limitation remained unchanged following treatment, although some samples taken from smokers with normal lung function appeared to increase expression following treatment, while some BEAS-2B cell samples exhibited a sharp decrease in expression which was offset by one sample which remained unchanged (Figure 6.4-8). Overall, fluticasone had no significant effect on the TWIST and Smad pathways in both primary and BEAS-2B cells.

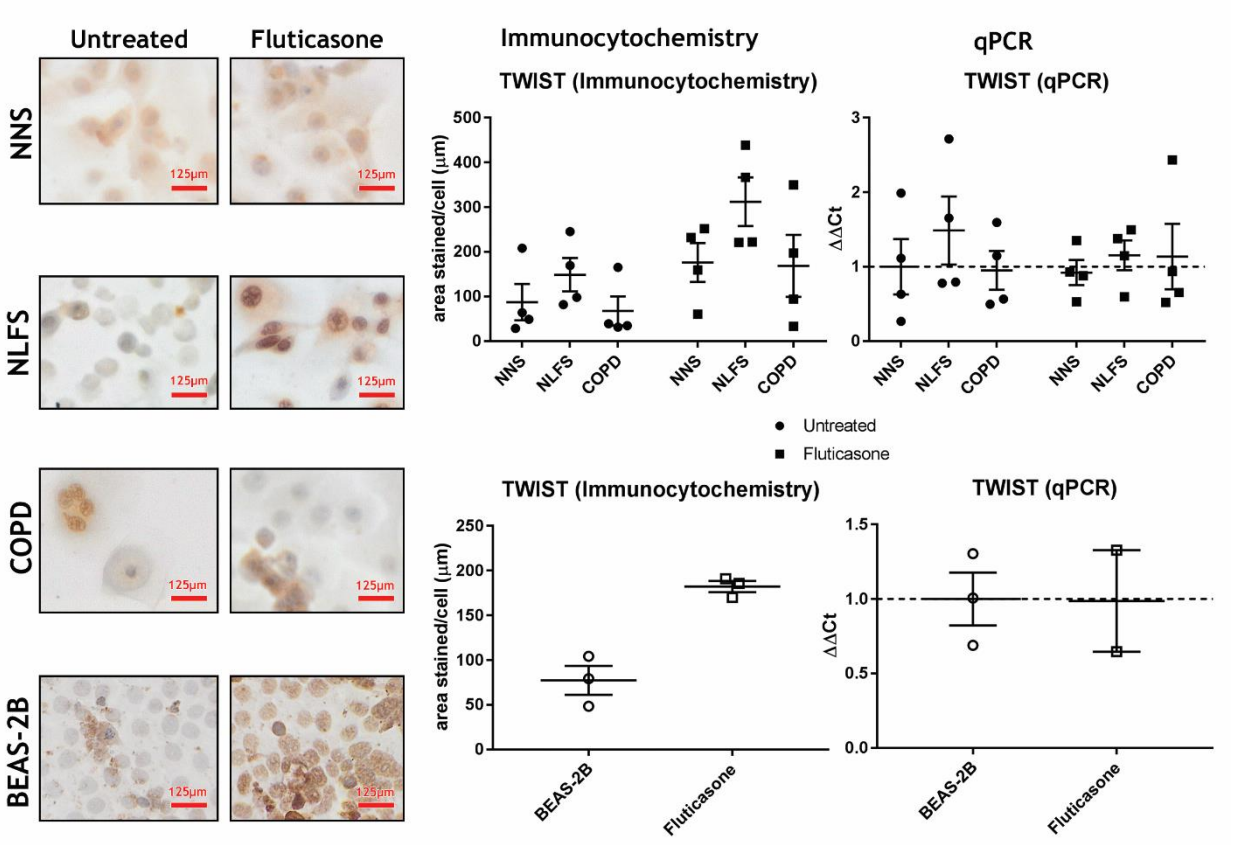

Figure 6.4-7: Expression of TWIST in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to fluticasone for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

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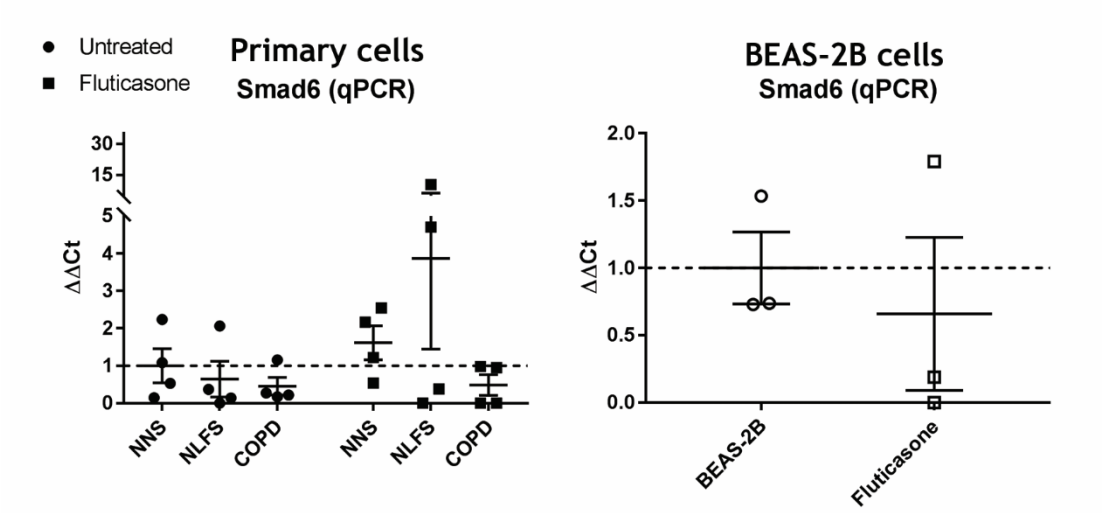

Figure 6.4-8: Expression of Smad6 in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by qPCR with and without exposure to fluticasone for 24 hours. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

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pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

6.4.2.1.4. Extracellular matrix remodelling

MMP2 expression was unaffected by fluticasone across all three groups of primary cells, although the more highly expressing BEAS-2B samples which initially expressed higher levelssample demonstrated adecrease in expression in response to treatment with fluticasone (Figure 6.4-9). Fluticasone had no effect on expression of collagen 1-α, either at the mRNA level or at the level of pro-collagen 1-α secreted into the cell culture supernatant in primary cells from non-smokers and smokers with normal lung function, as well as in BEAS-2B cells. However, aa subset of samples from people with airflow limitation appeared to demonstrate an increase in collagen 1-α expression following exposure to fluticasone. Fluticasone had no effect on extracellular matrix remodelling proteinsmay.

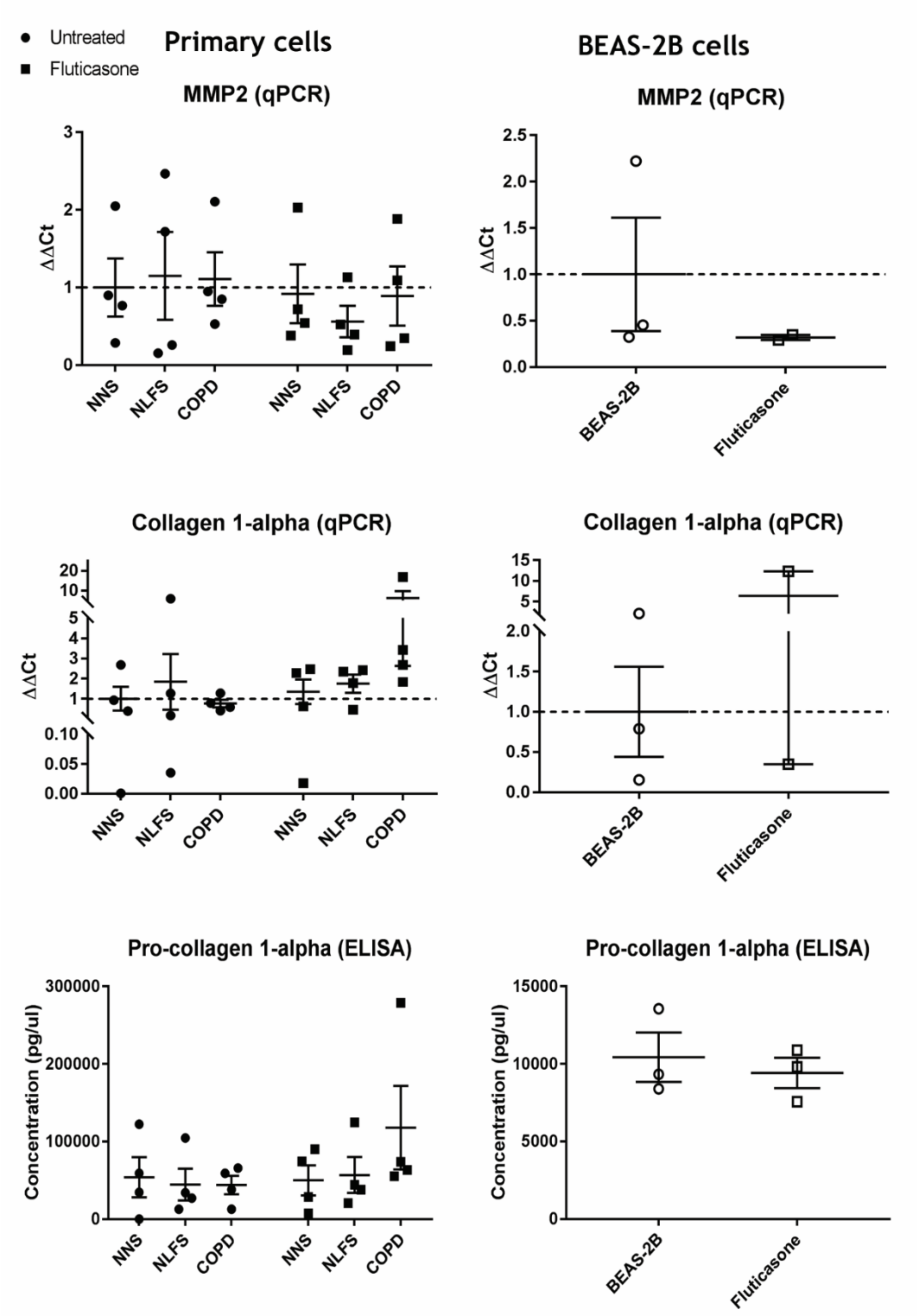

Figure 6.4-9: Expression of MMP2 and collagen 1-α in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) and immortalised BEAS-2B cells as measured by ELISA of cell culture supernatant and qPCR with and without exposure to fluticasone for 24 hours. *Data are represented as mean with SEM.* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

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6.4.2.2. TGF-β and CSE-induced EMT

6.4.2.2.1. Epithelial markers

The application of fluticasone to primary and BEAS-2B cells exposed to transforming growth factor-β1 (TGF-β) had no effect on expression of E-cadherin at either the protein or mRNA level (Figure 6.4-10). AA similar lack of effect was seen in both primary and BEAS-2B cells exposed to fluticasone and cigarette smoke extract (CSE).

Fluticasone appeared to have no effect on expression of cytokeratin in primary or BEAS-2B cells treated with either TGF-β or CSE (Figure 6.4-11), nor did it affect the expression of tight junction protein-1 (TJP1) in cells exposed to TGF-β or CSE (Figure 6.4-12). Overall, fluticasone had no effect on epithelial markers in primary possibly of BEAS-2B cells following exposure to TGF-β or CSE.

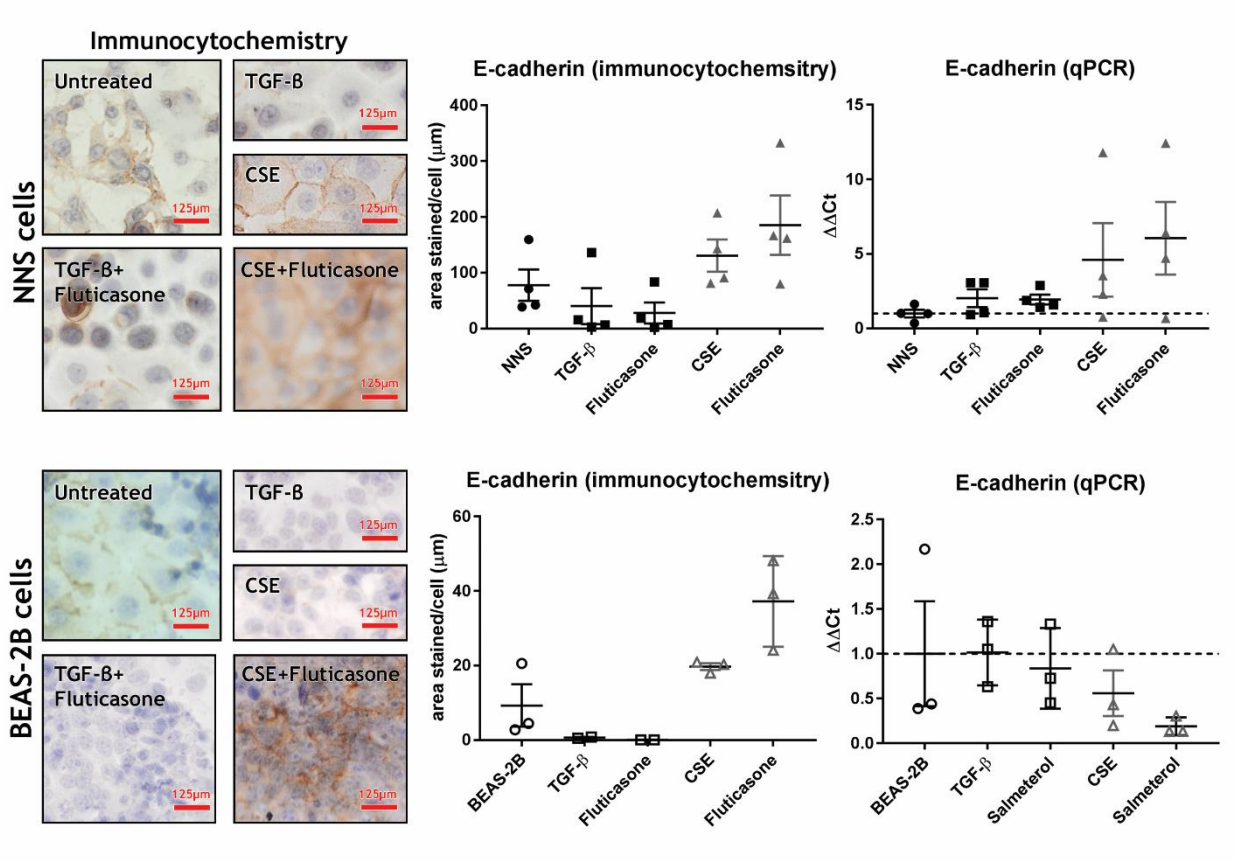

Figure 6.4-10: Expression of E-cadherin in non-smokers (NNS) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with fluticasone for 24 hours. Neither primary cells nor BEAS-2B cells treated with TGF-β exhibited a change in E-cadherin expression following exposure to fluticasone at either the protein or mRNA level. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

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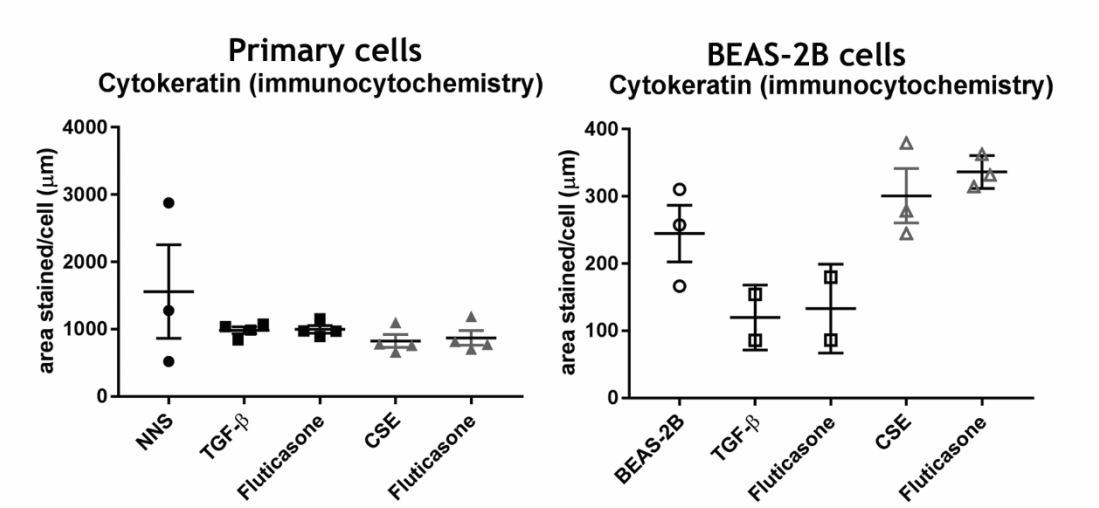

Figure 6.4-11: Expression of cytokeratin in non-smokers (NNS) and immortalised BEAS-2B cells as measured by immunocytochemistry with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with fluticasone for 24 hours. *Data are represented as mean with SEM. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).*

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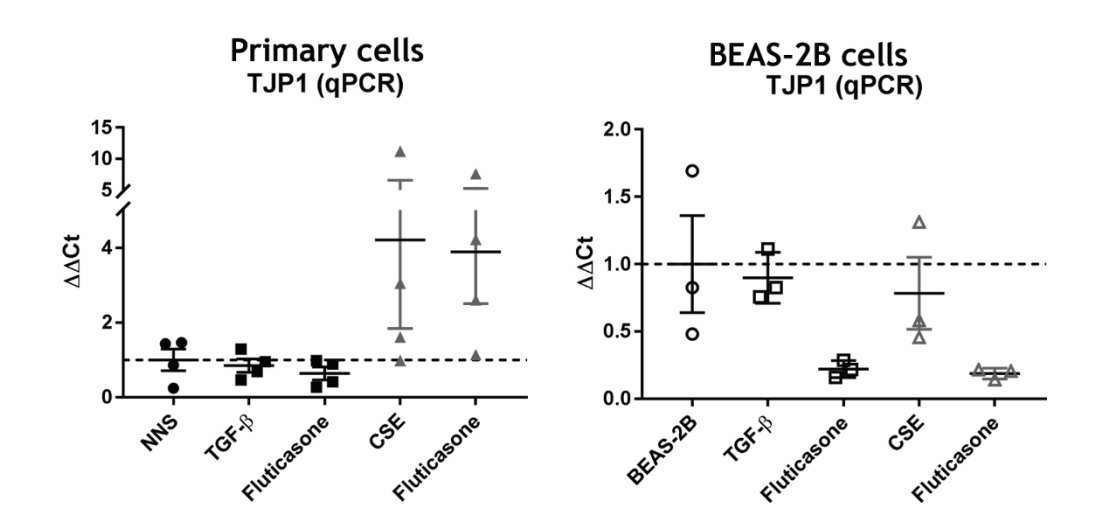

Figure 6.4-12: Expression of tight junction protein-1 (TJP1) in non-smokers (NNS) and immortalised BEAS-2B cells as measured by qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with fluticasone for 24 hours. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

6.4.2.2.2. Mesenchymal markers

In primary cells treated with either transforming growth factor-β1 (TGF-β) or cigarette smoke extract (CSE) fluticasone had no effect on the expression of N-cadherin at either the protein or mRNA level (Figure 6.4-13). At the protein and mRNA levels, BEAS-2B cells treated with TGF-β and fluticasone likewise appeared to have no change in expression of N-cadherin.potential expression,the change did not Similarlychanges

Fluticasone treatment had no effect on vimentin expression in either primary or BEAS-2B cells exposed to TGF-β (Figure 6.4-14). BEAS-2B cells treated with fluticasone and CSE likewise exhibited no change in their vimentin expression, however at both the protein and mRNA level primary cells exposed to CSE and fluticasone expressed increased levels of vimentin, with 3.9-fold higher levels of vimentin mRNA compared to untreated controls (one-way ANOVA $p = 0.0411$) and a trend towards increased expressioncompared to cells treated with CSE alone. Primary and BEAS-2B cells treated with fluticasone following exposure to either TGF-β or CSE exhibited no change in expression of S100A4 (Figure 6.4-15).

Overall, primary cells and BEAS-2B cells exhibited no response to fluticasone at baseline, however primary cells exposed to a combination of fluticasone and CSE increased expression of vimentin. BEAS-2B cells exposed to CSE were unaffected by CSE.

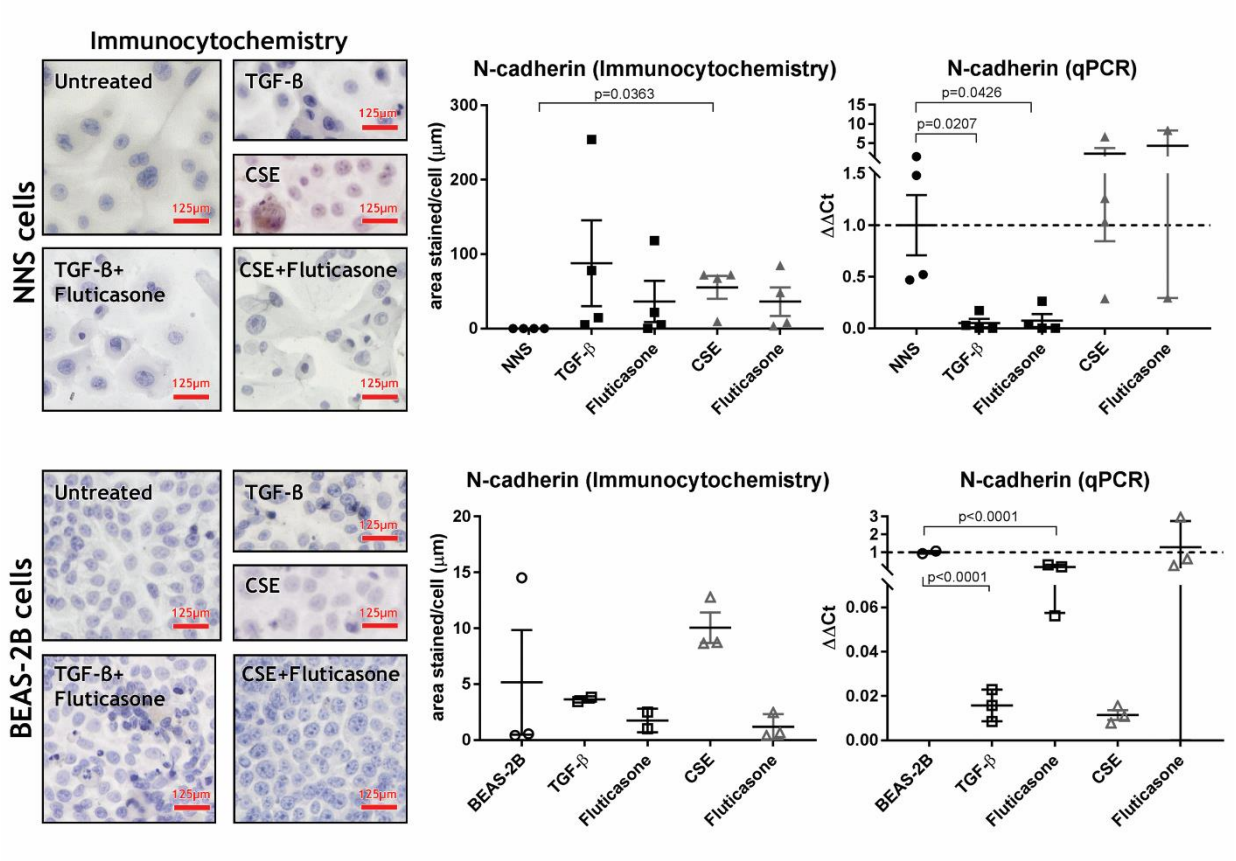

Figure 6.4-13: Expression of N-cadherin in non-smokers (NNS) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with fluticasone for 24 hours. Primary cells exposed to either TGF-β or CSE demonstrated no change in N-cadherin expression following exposure to fluticasone at either the mRNA or protein level. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

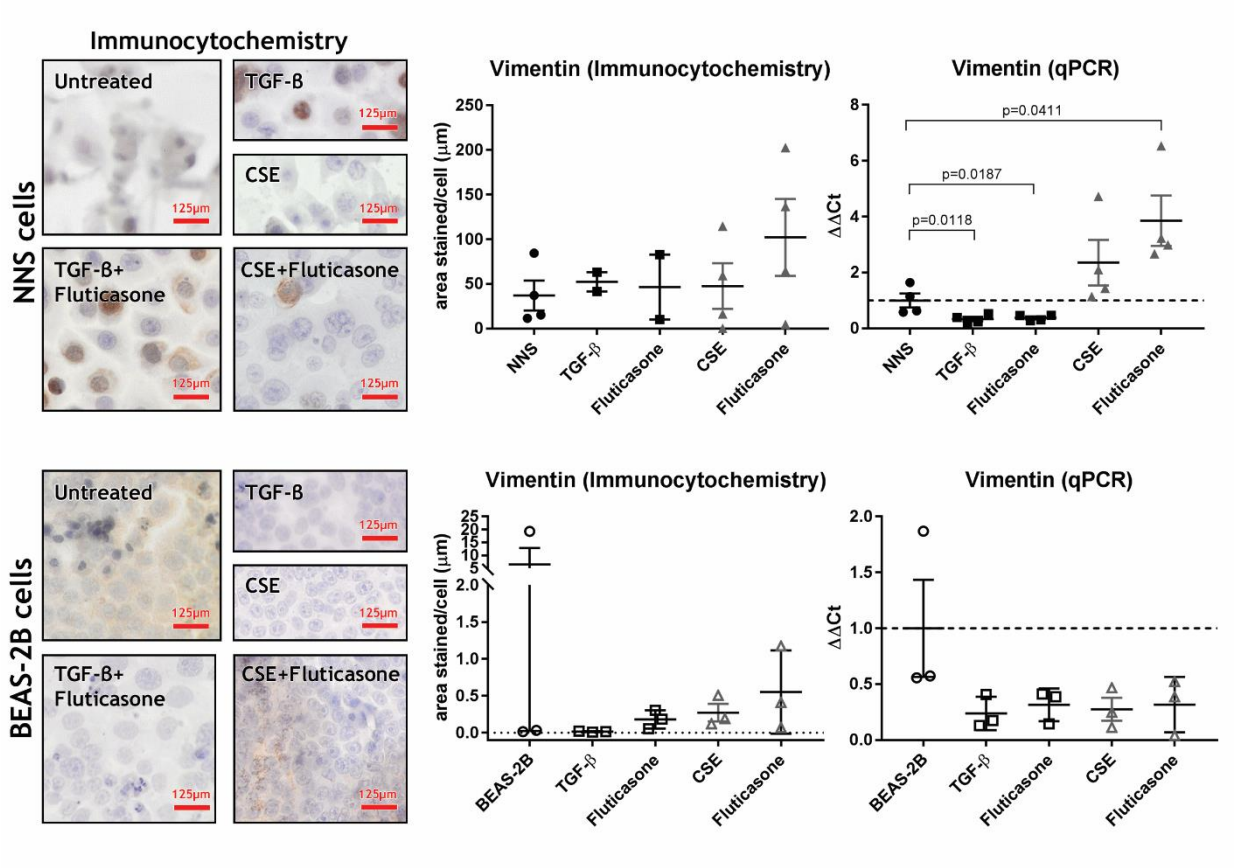

Figure 6.4-14: Expression of vimentin in non-smokers (NNS) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with fluticasone for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

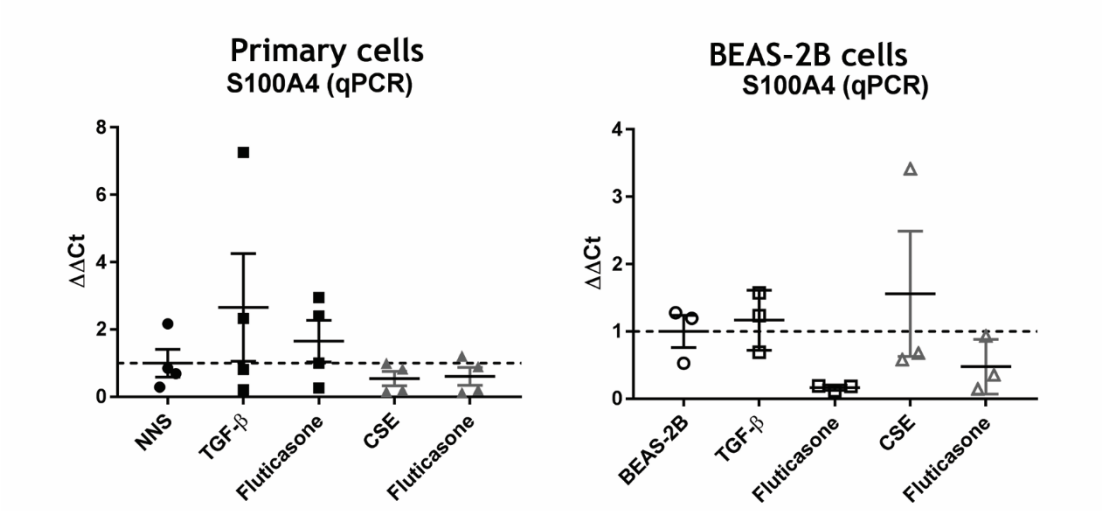

Figure 6.4-15: Expression of S100A4 in non-smokers (NNS) and immortalised BEAS-2B cells as measured by qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with fluticasone for 24 hours. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

6.4.2.2.3. Signalling molecules

Expression of TWIST at both the protein and mRNA levels in primary cells was unaffected by the combination of fluticasone and either transforming growth factor-β1 (TGF-β) or cigarette smoke extract (CSE) (Figure 6.4-16). While BEAS-2B cells did not exhibit changes in TWIST expression at the protein level following exposure to fluticasone and either TGF-β or CSE, at the mRNA level TWIST expression was increased5.0-fold by fluticasone when compared to cells exposed to TGF-β alone (one-way ANOVA p = 0.0360). BEAS-2B cells treated with fluticasone and CSE also trended towards increased expression of TWIST mRNA compared to cells treated with CSE alone, although this change did not reach statistical significance.

Primary and BEAS-2B cells treated with fluticasone following exposure to TGF-β demonstrateddemonstrated no change in expression of Smad6 (Figure 6.4-17). Overall, primary cells appeared to be insensitive to fluticasone with regards to the TWIST pathway, while BEAS-2B cells appeared to be activated at the mRNA level, even if these changes did not translate into protein-level changes. Smad6 was unaffected in both cell types by fluticasone combined with TGF-β or CSE.

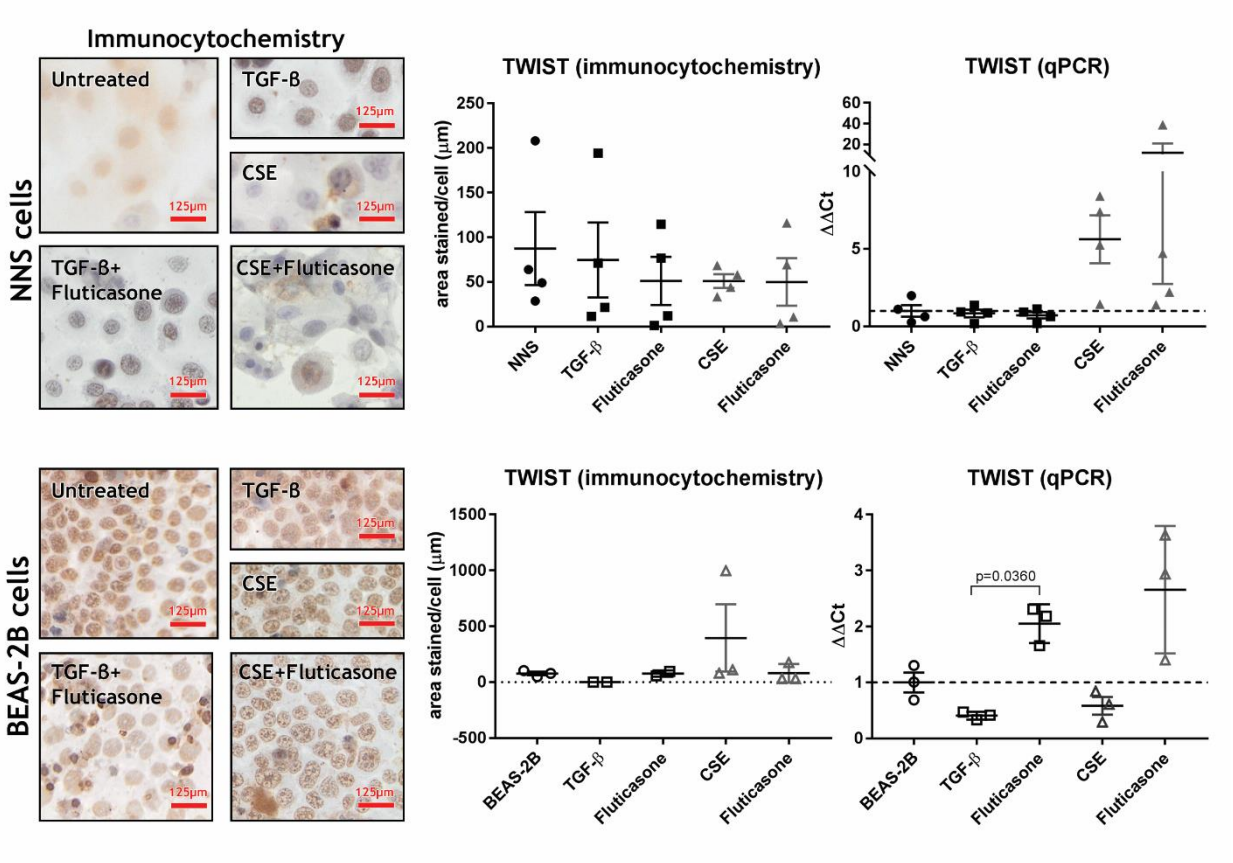

Figure 6.4-16: Expression of TWIST in non-smokers (NNS) and immortalised BEAS-2B cells as measured by immunocytochemistry and qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with fluticasone for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

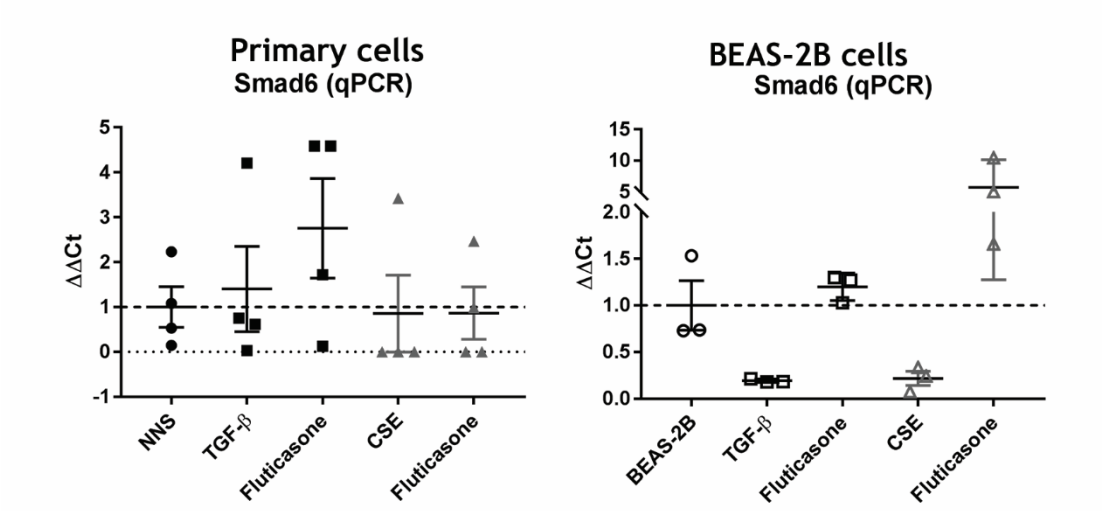

Figure 6.4-17: Expression of Smad6 in non-smokers (NNS) and immortalised BEAS-2B cells as measured by qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with fluticasone for 24 hours. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

6.4.2.2.4. Extracellular matrix remodelling

Fluticasone had no effect on MMP2 or collagen 1-α expression in primary or BEAS-2B cells treated with either transforming growth factor-β1 (TGF-β) or cigarette smoke extract (CSE) (Figure 6.4-18).

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

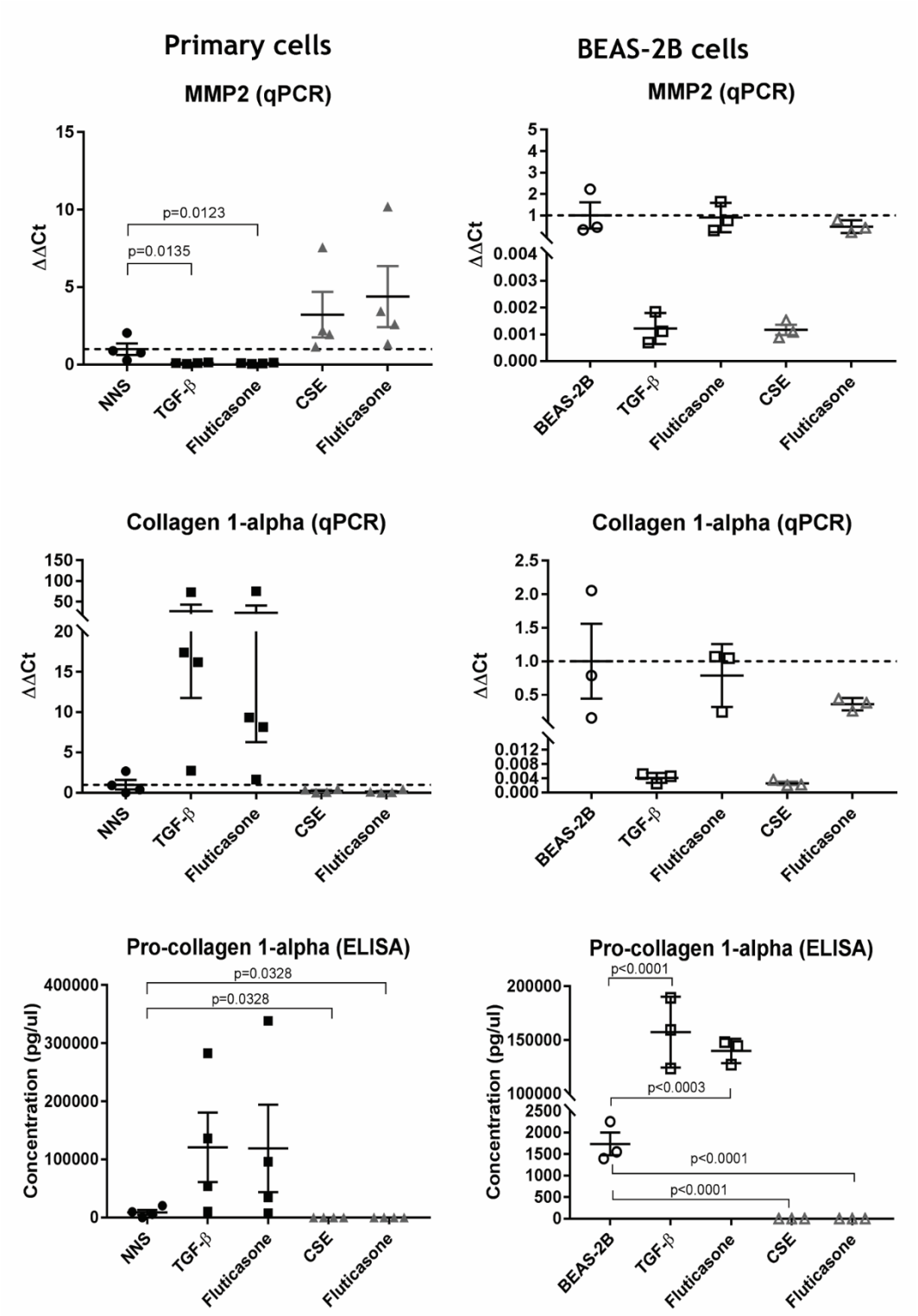

Figure 6.4-18: Expression of MMP2 and collagen 1-α in non-smokers (NNS) and immortalised BEAS-2B cells as measured by ELISA of cell culture supernatant and qPCR with and without exposure to TGF-β (72 hours) or CSE (4 hours) combined with fluticasone for 24 hours. *Data are represented as mean with SEM.* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are pHBECs, open shapes are BEAS-2B cells.*

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NNS *– non-smokers*
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LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

6.4.3. Discussion of the effects of fluticasone

Fluticasone had no effect on epithelial-mesenchymal transition (EMT) markers in unstimulated primary or BEAS-2B cells in this study. Fluticasone, in combination with cigarette smoke extract (CSE), appeared to promote a mesenchymal profile in both primary and BEAS-2B cells, although the change was only statistically significant in primary cells. Further work to confirm or deny the increased levels of N-cadherin in BEAS-2B cells is recommended, as the observed trend was not statistically significant, likely due to a lack of power and large variation between samples at baseline. InterestinglyInteresting, BEAS-2B cells appeared to express increased levels of N-cadherin in response to the combination of CSE and fluticasone, while primary cells expressed significantly increased levels of vimentin, once more demonstrating that BEAS-2B cells are likely an inaccurate model of EMT in normalnormal bronchial epithelial cells.

Despite the known inhibitory effect of the corticosteroid dexamethasone on epithelial cell proliferation and migration [305], in this study fluticasone had no effect on EMT-related markers. There are a number of possible explanations for this, from the possibility that fluticasone acts differently in epithelial cells to dexamethasone, to the possibility that although corticosteroids may affect epithelial cell migration they act through a non-EMT related pathway. Certainly the lack of signal in the TWIST and Smad6 pathways is not unexpected, as dexamethasone acts through the MAPK/Erk signalling pathway [305], which is an alternative non-Smad-dependant pathway for TGF-β signalling [87]. Further study focussing on the MAPK pathway would be recommended when building upon these results, however was not feasible within the current studies.

As with the other two drugs, fluticasone may only be able to exert a noticeable, real effect on activated epithelial cells underoing EMT, and future work with samples confirmed to be fully activated by TGF-β and/or CSE is highly recommended.

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

6.4.4. Conclusions on the effect of fluticasone

BEAS-2B cells exposed to cigarette smoke extract (CSE) appeared to respond differently to fluticasone than primary cells taken from non-smokers, although further work is required to confirm this. The differences in response makes BEAS-2B cells undesirable as models for bronchial epithelial cells' responses to corticosteroids in activated bronchial epithelial cells, although they appeared to accurately represent untreated primary cells' responses. Fluticasone hadhad no significant effect on EMT-related marker expression in unstimulated cells, however the combination of fluticasone and CSE promoted an increasedpromote a mesenchymal phenotype, which suggests that fluticasone may not be an ideal treatment for COPD in cases where the patient does not cease smoking.

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
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pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

6.5. Combination therapy

6.5.1. Introduction

Due to the complicated nature of diseases such as COPD, it can be beneficial to give patients multiple drugs concurrently, and thus target a number of different contributing pathways. In addition, some drug combinations produce synergistic effects, which is to say that in combination they produce a more marked effect than would be expected by combining their individual effects. For example, the effect of tiotropium is enhanced when combined with a corticosteroid such as formoterol [306]. In the GOLD guidelines for the treatment and management of COPD, triple therapy is noted as a step up from dual therapy with a long-acting β-agonist (LABA) and either an inhaled corticosteroid or long-acting muscarinic antagonist (LAMA) [208]. There are a variety of triple-powder inhalers available, and the combination of tiotropium bromide, fluticasone and salmeterol has been examined in a number of trials.

The EAGLE study demonstrates that tiotropium (a LAMA) combined with salmeterol (a LABA) and fluticasone (a corticosteroid) provides an increase in airway conductance and an improvement in both FEV1 and residual volume compared to fluticasone and salmeterol without tiotropium [301]. The combination of salmeterol, tiotropium and fluticasone has been shown to improve lung function indices such as forced expiratory volume in one second (FEV_1) and bronchodilation, as well as reducing dyspnoea and reducing the need for rescue mediation [302, 303]. Similar results hold true for other combinations of LAMA/LABA/corticosteroid triple therapy [304]. As discussed in previous sections in this chapter, salmeterol, tiotropium and fluticasone all have confirmed or likely actions in the bronchial epithelium (sections 6.2.1, 6.3.1 and 6.4.1), therefore the action of the three drugs in combination on the epithelium is worth investigating.

6.5.2. Results

6.5.2.1. Epithelial markers

The combination of salmeterol, tiotropium and fluticasone had no effect on E-cadherin levels in any of the three primary cell groups at the protein level, however ithowever it did appear to reduce variability between samples, although this could not be confirmed via statistical analysis (Figure 6.5-1). Cytokeratin expression was unaffected by the combination of drugs (Figure 6.5-2), as was tight junction protein-1 expression (Figure 6.5-3). Overall, the drugs had no effect on epithelial markers in any of the cell groups.

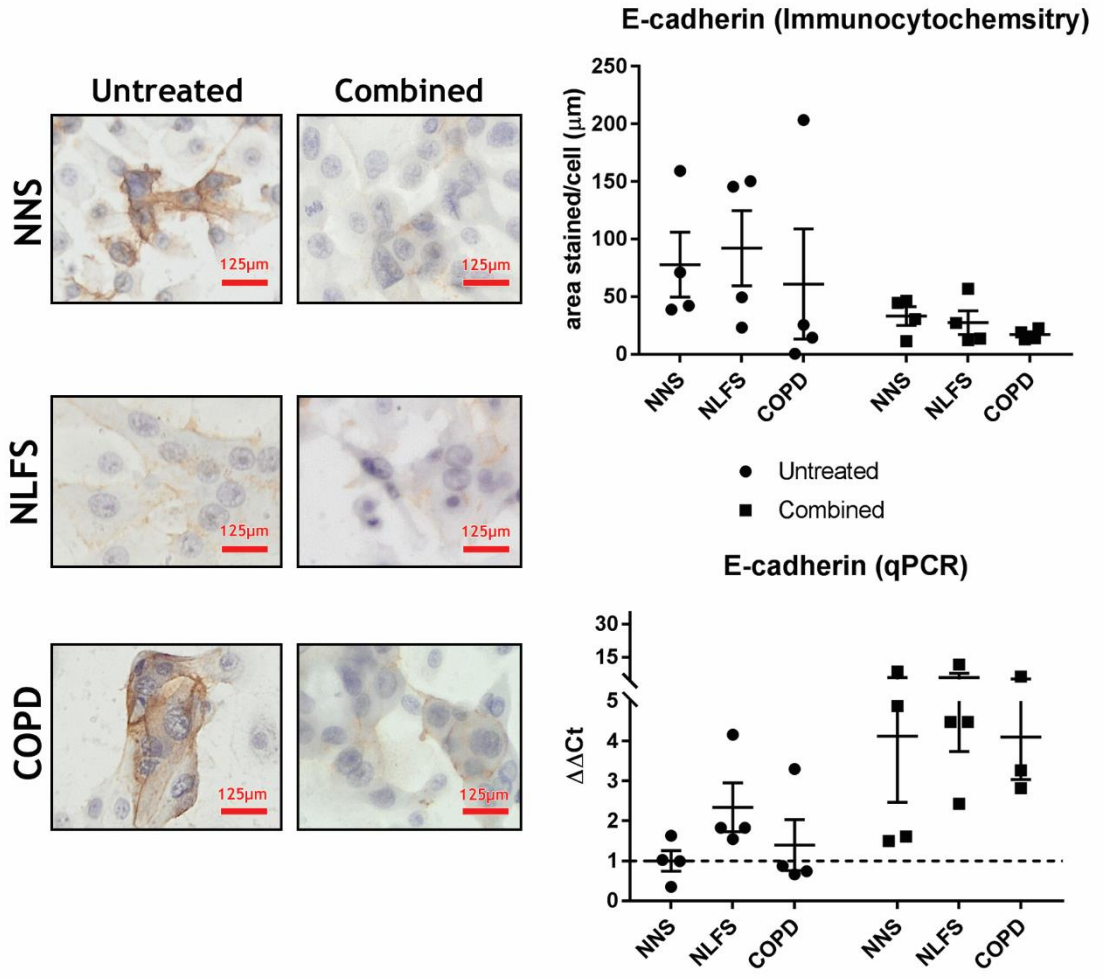

Figure 6.5-1: Expression of E-cadherin in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) cells as measured by immunocytochemistry and qPCR with and without exposure to a combination of salmeterol, tiotropium and fluticasone for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer.* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Useful abbreviations

NNS *– non-smokers*
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LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

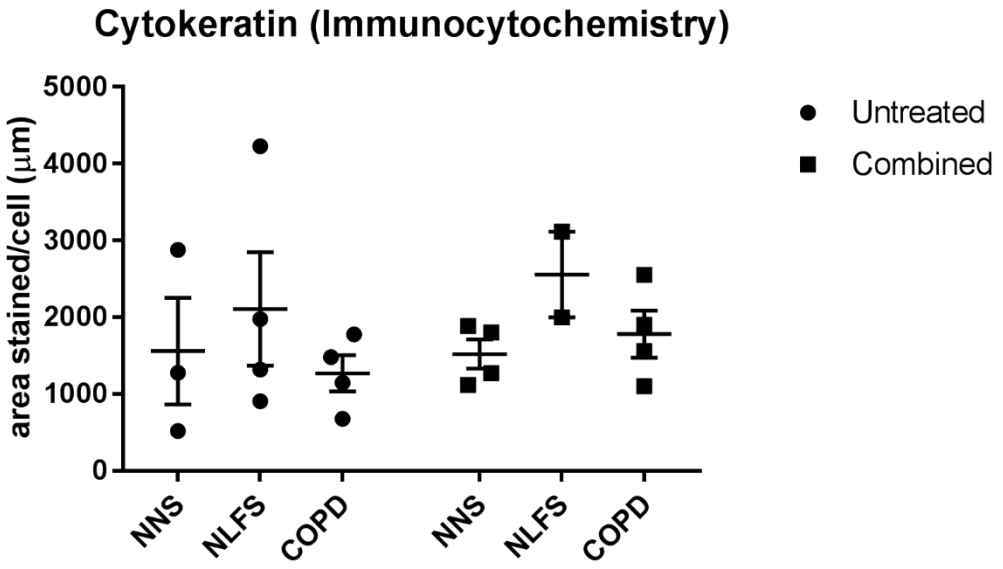

Figure 6.5-2: Expression of cytokeratin in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) cells as measured by immunocytochemistry with and without exposure to a combination of salmeterol, tiotropium and fluticasone for 24 hours. *Data are represented as mean with SEM. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer.*

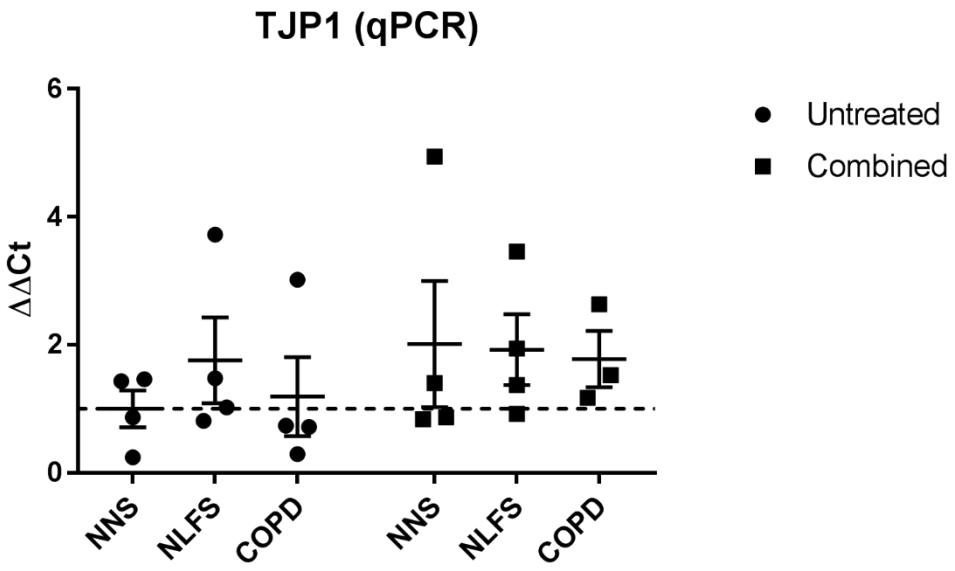

Figure 6.5-3: Expression of tight junction protein-1 (TJP1) in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) cells as measured by qPCR with and without exposure to a combination of salmeterol, tiotropium and fluticasone for 24 hours. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

6.5.2.2. Mesenchymal markers

Application of the combination of salmeterol, tiotropium and fluticasone to primary cells appeared to increaseincrease expression of N-cadherin in a subsetsubset of the samples taken from non-smokers while also reducing expression in the smokers with normal lung function cells which expressedexpressed high levels of N-cadherin protein at baseline (Figure 6.5-4). Despite these trendspossible changes, no overall effect on N-cadherin protein expression was observed in primary cells treated with the three drugs. The mRNA expression of N-cadherin remained unchanged following treatment in cells from non-smokers and smokers with normal lung function and the drugs hadhad no effect on the protein level expression of N-cadherin in cells from people with airflowairflow limitation. No comment can be made on the mRNA levels in cells from people with airflow limitation due to only having a single post-treatment datum point.

Vimentin expression was unchanged by treatment at the protein level, except for reducing expression in a single current smokers' sample which initially expressed high levels of vimentin (Figure 6.5-5). At the mRNA level, there appeared to be a subset of cells from both the smokers with normal lung function and people with airflow limitation which increased expression of vimentin in response to the drug combination, however others remained unchanged. Non-smokers, however, appeared to increase expression of vimentin mRNA in response to the drugs and, if the three untreated groups are combined into a single 'baseline' group and compared to the treated non-smokers' cells then the 16.3-fold increase reaches statistical significance (Welch's t-test $p = 0.0349$). S100A4 expression remained unchanged in cells from smokers with normal lung function following treatment, however one individual from each the non-

smoker and people with airflow limitation group exhibited a sharp increase in expression after exposure (Figure 6.5-6).

Overall, the combination of salmeterol, tiotropium and fluticasone appeared, with the exception of the increased mRNA levels of vimentin in non-smokers' cells, to have no effect on the expression of mesenchymal markers in primary cells.

Useful abbreviations

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COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

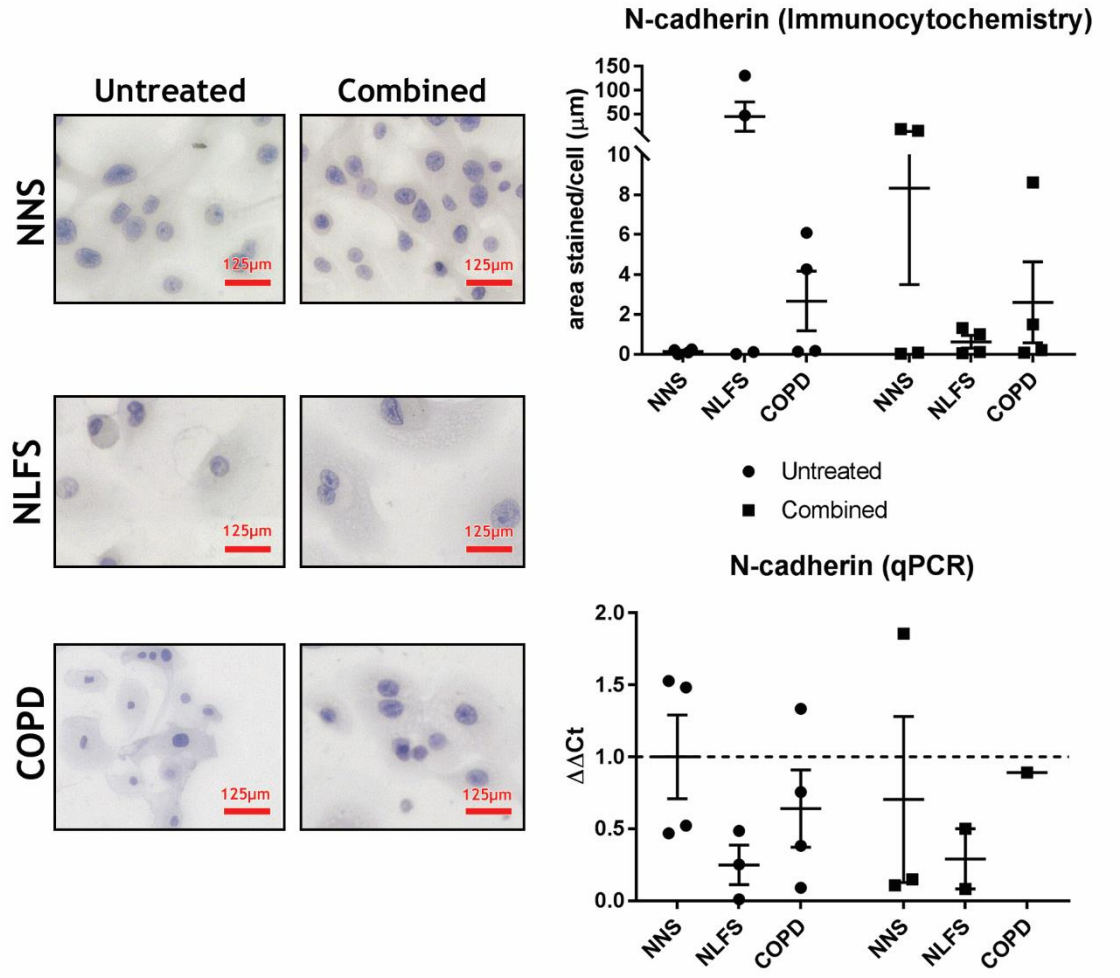

Figure 6.5-4: Expression of N-cadherin in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) cells as measured by immunocytochemistry and qPCR with and without exposure to a combination of salmeterol, tiotropium and fluticasone for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer.* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

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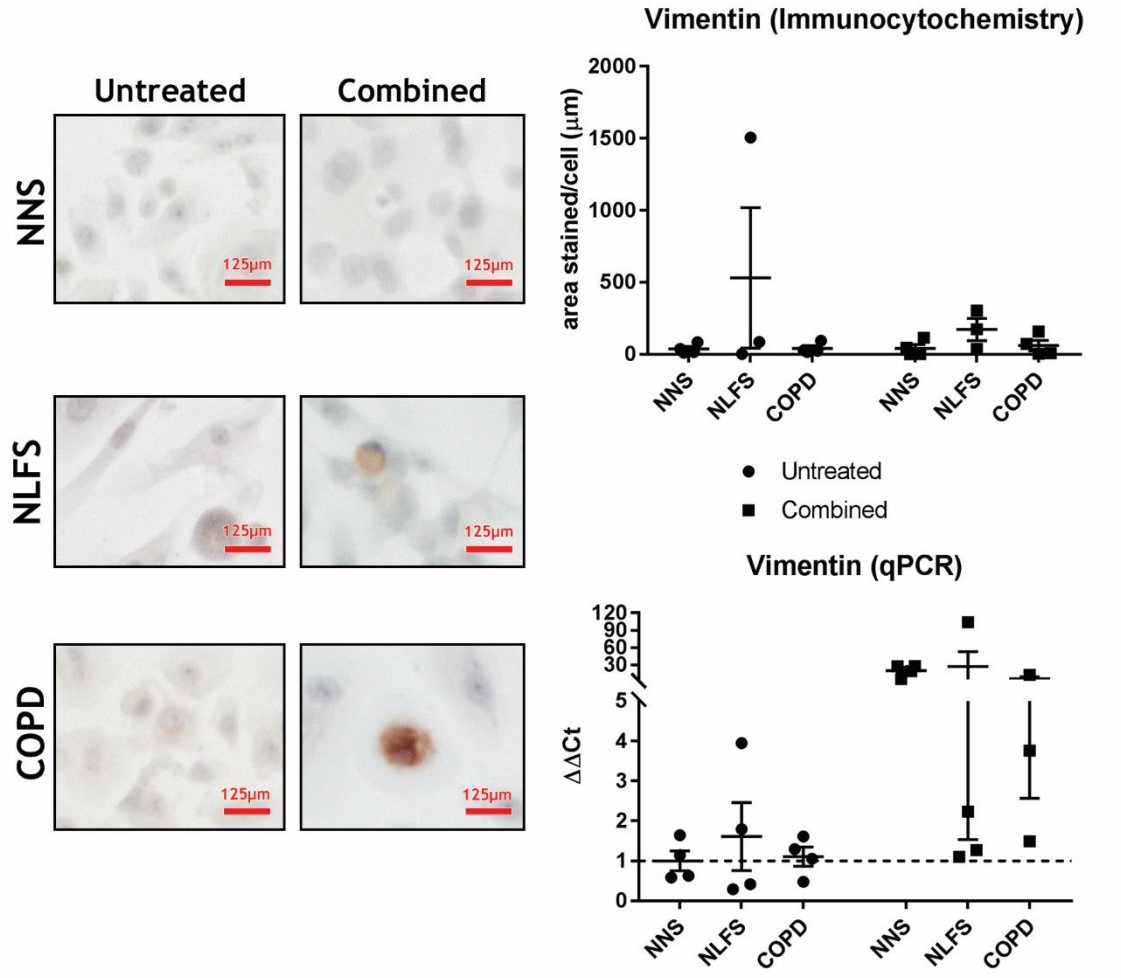

Figure 6.5-5: Expression of vimentin in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) cells as measured by immunocytochemistry and qPCR with and without exposure to a combination of salmeterol, tiotropium and fluticasone for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single.* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

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CSE *– cigarette smoke extract*

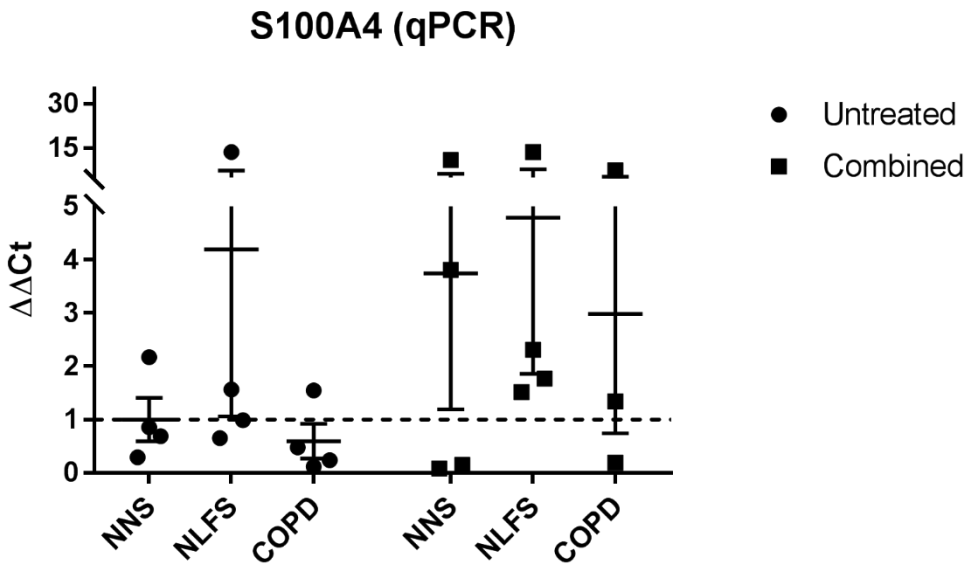

Figure 6.5-6: Expression of S100A4 in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) cells as measured by qPCR with and without exposure to a combination of salmeterol, tiotropium and fluticasone for 24 hours. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

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pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

6.5.2.3. Signalling molecules

At both the protein and mRNA levels expression of TWIST was beunaffected by exposure to with the combination of salmeterol, tiotropium and fluticasone across all three groups of primary cells (Figure 6.5-7). In contrast, cells from in non-smokers and smokers with normal lung function exhibited reduced appeared to cause a reduction in variability in Smad6of expression between samples following treatment, as well as potentially decreasing overall expression, although this change did not reach statistical significance (Figure 6.5-8).) Smad6 expression in cells taken from people with airflow limitation were unaffected by the drug cocktail. Overall,the three drugs in combination did not appear to affect the expression of either the Smad or TWIST signalling pathways.

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COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

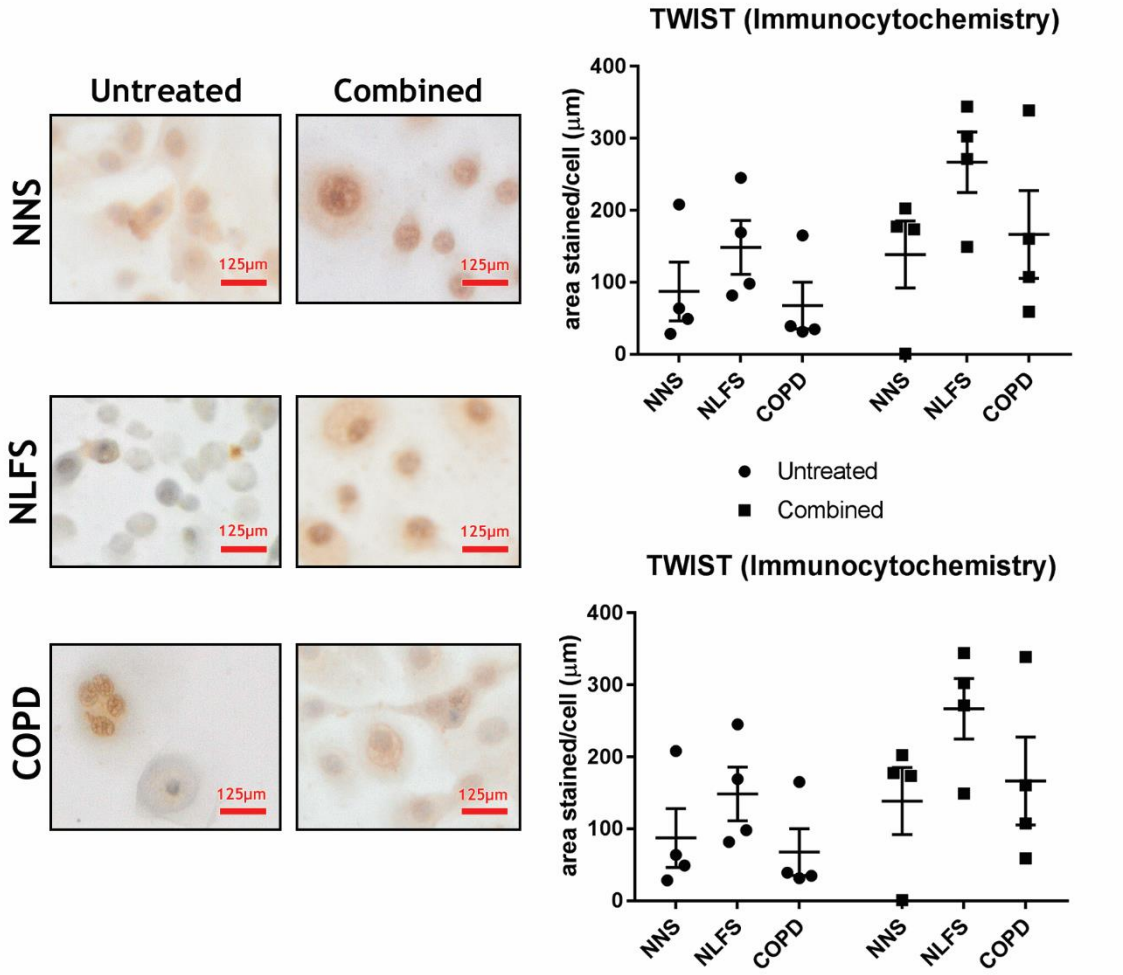

Figure 6.5-7: Expression of TWIST in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) cells as measured by immunocytochemistry and qPCR with and without exposure to a combination of salmeterol, tiotropium and fluticasone for 24 hours. *Data are represented as mean with SEM.* ***Immuno:*** *In immunocytochemical images cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin. Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer.* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

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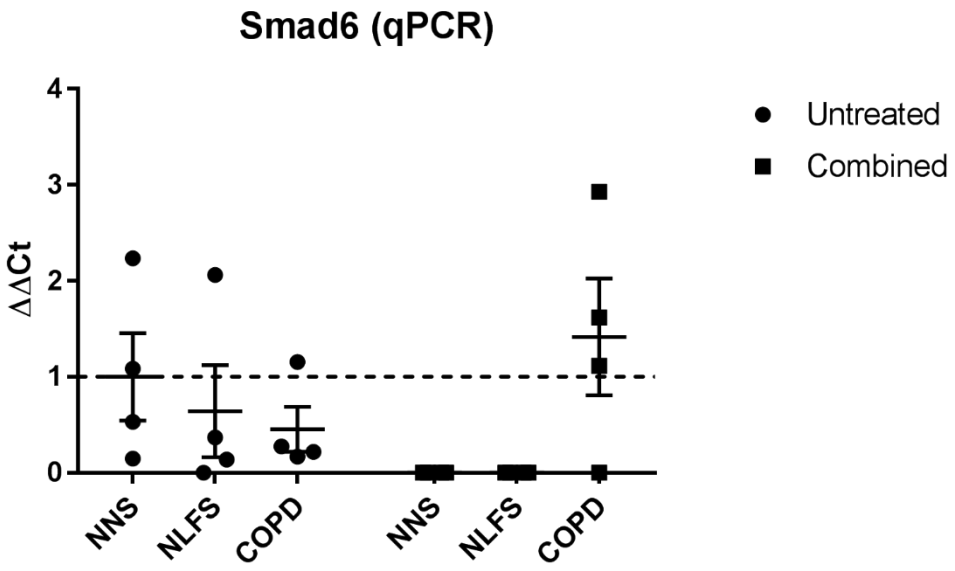

Figure 6.5-8: Expression of Smad6 in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) cells as measured by qPCR with and without exposure to a combination of salmeterol, tiotropium and fluticasone for 24 hours. *Data are represented as mean with SEM. Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

6.5.2.4. Extracellular matrix remodelling

Exposure to the increased MMP2 expressiona subset of samples across the three primary cell groups, although there was no significant overall change in MMP2 expression (Figure 6.5-9). The drugs also appeared to have no effect on collagen 1-α, both at the mRNA level and as measured by quantification of pro-collagen 1-α in the culture supernatant, although it appeared to appeared to induce increased variability in samples taken from people with airflow limitation. Overall, the combination of salmeterol, tiotropium and fluticasone havehave no effect on either MMP2 or collagen 1-α expression.

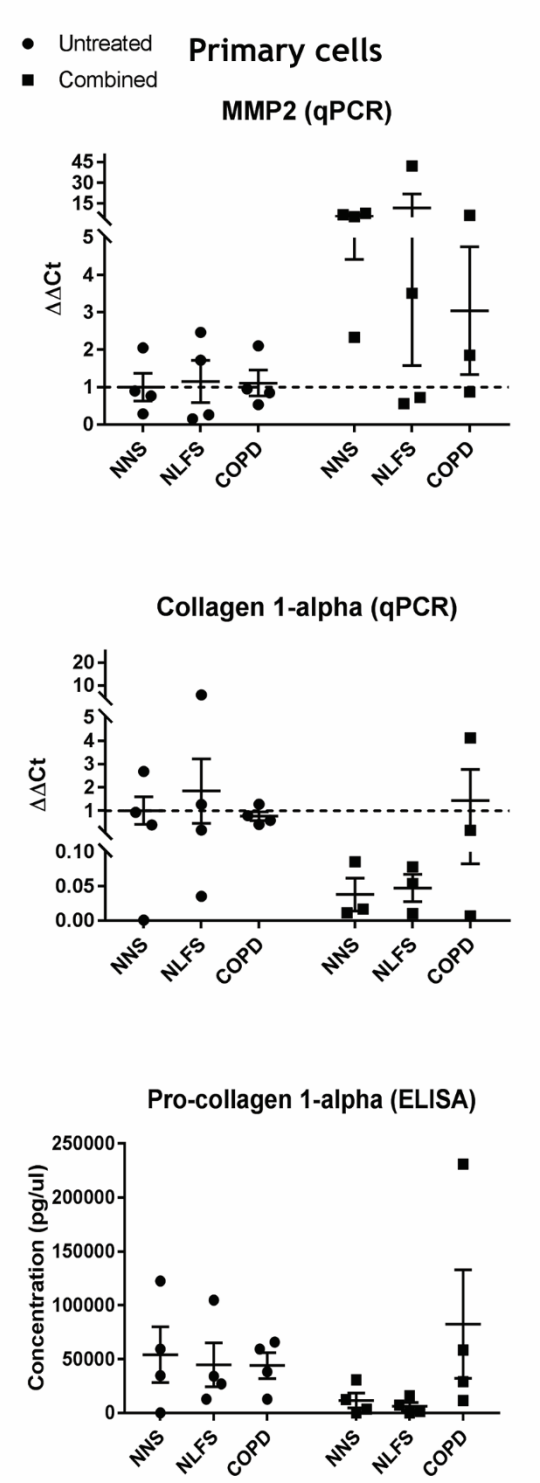

Figure 6.5-9: Expression of MMP2 and collagen 1-α in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) cells as measured by ELISA of cell culture supernatant and qPCR with and without exposure to a combination of salmeterol, tiotropium and fluticasone for 24 hours. *Data are represented as mean with SEM.* ***qPCR:*** *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. The dashed line indicates the expression of the untreated cells.*

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

6.5.3. Discussion of the effects of combined drug therapy

The combination of salmeterol, tiotropium and fluticasone appeared to have no effect on markers of epithelial-mesenchymal transition (EMT) in primary cells, with the exception of vimentin, which was increased at the mRNA level in non-smokers' cells only. However, the changes observed in vimentin were almost identical to the effects of tiotropium alone (6.3.2), suggesting that the combination of the three drugs had no additive or synergistic effect on the cells with regards to EMT related markers. The drug cocktail also had no effect on the expression of the extracellular matrix remodelling markers MMP2 or collagen 1-α.

DespiteDespite the presence of salmeterol in the mixture, the TWIST pathway appeared to be unaffected, which was unexpected [173, 234, 275] however appeared consistent within the results of this thesis. It was unclear why TWIST was not upregulated in this study, and while the combination of sample sample sizes and large variation between samples may have contributed, the activity of the drugs should be confirmed prior to use in future studies.

6.5.4. Conclusions on the effects of combined drug therapy

The combination of salmeterol, tiotropium and fluticasone had no additive or synergistic effects on the primary cells compared to the individual application of the drugs. Thechanges observed in the expression of vimentin were similar to the effects of tiotropium alone.

7. PAFr expression in chronic airflow limitation

7.1. Introduction

7.1.1. Bacterial infection and PAFr in COPD

People with COPD experience higher rates of chronic and acute bacterial infections than those without COPD [42, 44], and bacterial infections are commonly seen in acute exacerbations [45]. The most commonly seen bacteria in COPD airways are *Haemophilius influenzae, Staphylococcus aureus, Streptococcus pneumoniae* or *Pseudomonas aeruigonsa* [42, 47, 48], with *H. influenzae* being the most predominant and occurring in 20-30% of exacerbations [44]. The reasons these bacteria are able to colonise the airways so effectively in COPD and the mechanisms by which they persist are currently unclear, although recent work has shed light on the role of attachment molecules such as platelet-activating factor receptor (PAFr). Attachment to the airway epithelium has been known for some time to be a key step in the establishment and persistence of bacterial infection [51], and the mechanism behind how these bacterial species attach outside of COPD has been studied. It is thought that upregulation and overexpression of adhesion molecules, such as PAFr, may be an additional feature of an activated or irritated epithelial layer, in addition to processes like epithelial-mesenchymal transition.

H. influenzae, *Streptococcus pneumonia* and *P. aeruginosa* express phosphorylcholine, abbreviated as ChoP, which they utilise as a host-attachment molecule [49, 50, 52]. ChoP is an oligosaccharide which, among many other functions, is one of the major active groups of platelet-activating factor (PAF) [107]. Bacteria which express ChoP are capable of using this similarity to attach to cells via PAFr [49, 50, 52]. This of course raises the question of whether

this mechanism is important in the airways; previous work has demonstrated that the airway epithelium expresses PAFr, albeit at low levels in healthy non-smokers' airways [199, 245, 246]. Importantly, PAFr expression is increased in the airways of people with COPD and in smokers [199, 245, 246] which suggests that it, and CHoP, may play a role in the increased prevalence of bacterial infection in COPD. Looking at the functional aspects of the relationship between PAFr and CHoP, previous work with normal, immortalised bronchial epithelial cells has shown that they express low levels of PAFr when grown in culture [177, 199]. However, if bronchial epithelial cells are stimulated with cigarette smoke, then the expression of PAFR is increased and *H. influenzae* are able to attach more readily to these activated cells [49, 50, 199]. This suggests a potentially key role for PAFr in the pathology of COPD and makes it attractive for further examination. Insofar this study examinedxamined PAFr, itwas as an additional marker for epithelial activation rather than as a bacterial adhesion molecule, since the microbiological aspects were outside the scope of this work.

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

7.1.2. Viral infection and ICAM-1 in COPD

Viral infections, similarly to bacterial infections, are known to be associated with 23-39% of acute exacerbations of COPD, although there is a suggestion they may precede as many as 64% of exacerbations and are thought to promote the likelihood and severity of the events [305]. While the precise pathways and mechanisms behind how they trigger exacerbations are largely unknown, their increased prevalence in COPD is of some concern, given this association. The most common viral pathogen seen in COPD and acute exacerbations is *Rhinovirus*, which is responsible for the common cold, and occurs in 20-58% of virus-associated exacerbations [44, 305]. *Rhinovirus* are known to adhere to cells and gain entry via a transmembrane glycoprotein called intercellular adhesion molecule-1 (ICAM-1) [306], and the expression of ICAM-1 is known to be upregulated in COPD airways [243, 247, 248, 251, 252]. As with PAFr, the scope of this study did not allow examination of the functional aspects of ICAM-1 with regards to viral adhesion, but instead looked at it as a marker of dysregulated epithelial function in people with airflowairflow limitation.

Looking at both PAFr and ICAM-1, the question arises whether upregulation of these molecules in occurs in primary bronchial epithelial cells activated by stimulus such as transforming growth factor-β1 and cigarette smoke extract. Additionally, examination of the degree of change and a comparison of primary cells to immortalised cells could reveal if immortalised cell culture models should be utilised to test the effects of potential therapies, and the answer could help ascertain the validity of the BEAS-2B immortalised cell line as a model for airway epithelial cells.

7.1.3. Chapter aims

The aims of this chapter were as follows:

- To determine the baseline level of expression of PAFr and ICAM-1 in primary bronchial epithelial cellsisolated from non-smokers, smokers with normal lung function and people with airflow limitation and to compare levels of PAFr and ICAM-1 expression between these three groups.
- To compare the expression of PAFr in primary bronchial epithelial cells to the expression of PAFr in the commercially available, immortalised bronchial epithelial BEAS-2B cell line.
- To examine the effect transforming growth factor β1 on the expression of PAFr in primary epithelial cells and immortalised bronchial epithelial cells.
- To examine the effect of cigarette smoke extract on the expression of PAFr in primary and immortalised bronchial epithelial cells.
- To examine the effects of a long-acting β-agonist (salmeterol), a long-acting muscarinic antagonist (tiotropium) and a corticosteroid (fluticasone) on primary and immortalised bronchial epithelial cells at baseline with regards to PAFr expression and, in primary epithelial cells only, ICAM-1 expression.

  To examine the effects of a combination of a long-acting β-agonist (salmeterol), a long-acting muscarinic antagonist (tiotropium) and a corticosteroid (fluticasone) on PAFR expression in primary bronchial epithelial cells following exposure to TGF-β or CSE.

  - **Hypothesis:** The three drugs will decrease expression of PAFr and ICAM-1, with salmeterol being effective in cells exposed to cigarette smoke extract and

tiotropium bormide monohydrate being more effective in transforming growth factor-β1 exposed cells.

Hypothesis: Bronchial epithelial cellsCells isolated from people with airflow limitation and BEAS-2B cells will exhibit increased levels of the microbial adhesion molecules PAFr and ICAM-1 compared to bronchial epithelial cells from non-smokers. Smokers' cells will exhibit intermediate levels of PAFr and ICAM-1. Transforming growth factor-β1 and cigarette smoke extract will increase expression of PAFr in both primary cells isolated from non-smokers and in immortalised bronchial epithelial cells. The three drugs, salmeterol, tiotropium, and fluticasone will decrease expression of PAFr and ICAM-1.

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

7.2. Results

7.2.1. PAFr

7.2.1.1. PAFr expression in pHBECs from non-smokers, smokers with normal lung function and people with airflow limitation and BEAS-2B cells

At both the protein (Figure 7.2-1) and the mRNA level (Figure 7.2-2), there was no difference in the expression of PAFr between cells taken from non-smokers, smokers with normal lung function and people with airflow limitation. Salmeterol had no significant effect on either the protein or mRNA level expression of PAFr in the primary cells, nor did tiotropium or fluticasone (Figure 7.2-1 and Figure 7.2-2). However, there was a suggestion that the combination of salmeterol, tiotropium and fluticasone may increaseincrease expression of PAFr protein and mRNA in primary cells taken from non-smokers (Figure 7.2-1). Indeed, if the untreated samples from the non-smokers, smokers with normal lung function and people with airflow limitaion were combined into a single 'baseline' group then the 106-times increase caused by the combination of drugs reached statistical significance (one-way ANOVA p = 0.0034). A smaller, non-significant 9-fold increase in PAFr expression was also seen at the mRNA level in non-smokers' cells in response to all three drugs (Figure 7.2-2).

BEAS-2B cells expressed 5.8 times more PAFr at the protein level than cells from non-smokers (Figure 7.2-3) and 93 times more PAFr at the mRNA level (Figure 7.2-4), although the number of samples was insufficient to allow formal statistical tests to detect a significant difference. Salmeterol, tiotropium or fluticasone had no effect on the expression of PAFr at the protein or mRNA level in BEAS-2B cells (Figure 7.2-5 and Figure 7.2-6). Overall, neitherneither smoking nor airflow limitation hadappeared to have an effect on PAFr expression, and

salmeterol, fluticasone or tiotropium alone had no effect on PAFr levels. A combination of all three drugs caused PAFr expression to increase, however only in non-smokers. BEAS-2B cells appeared to express higher levels of PAFr than cells from non-smokers, although like the primary cells they did not respond to any of the drugs.

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
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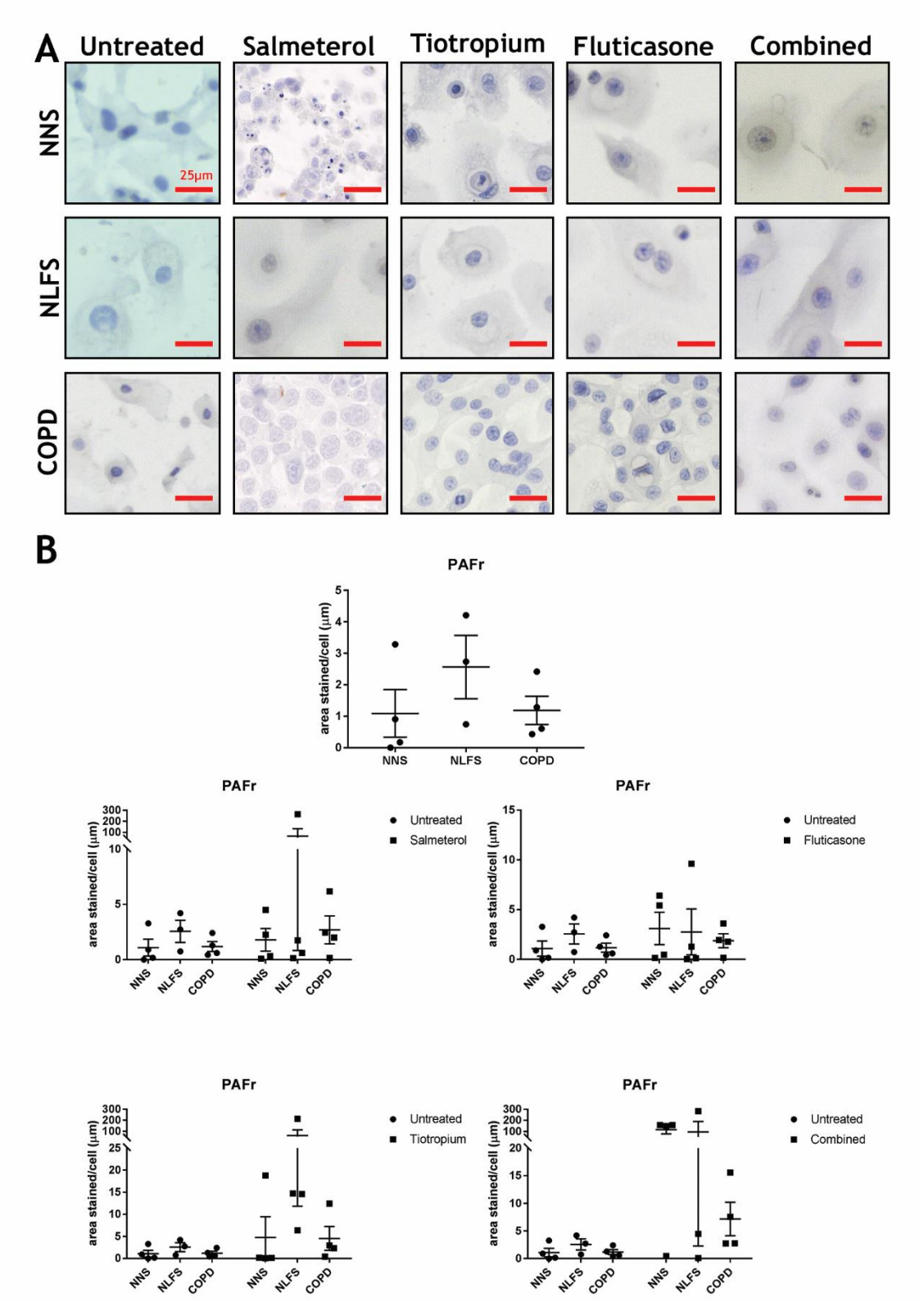

Figure 7.2-1: Baseline PAFr protein expression in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) as measured by immunocytochemistry with and without exposure to drugs. *Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer.*

A: *Immunocytochemical images of cells from non-smokers, smokers with normal lung function and people with airflow limitation (COPD). Cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin.* ***B:*** *Graphical representation of the area stained by immunocytochemistry quantified in the three groups. Data are represented as mean with SEM.*

Useful abbreviations

NNS *– non-smokers*
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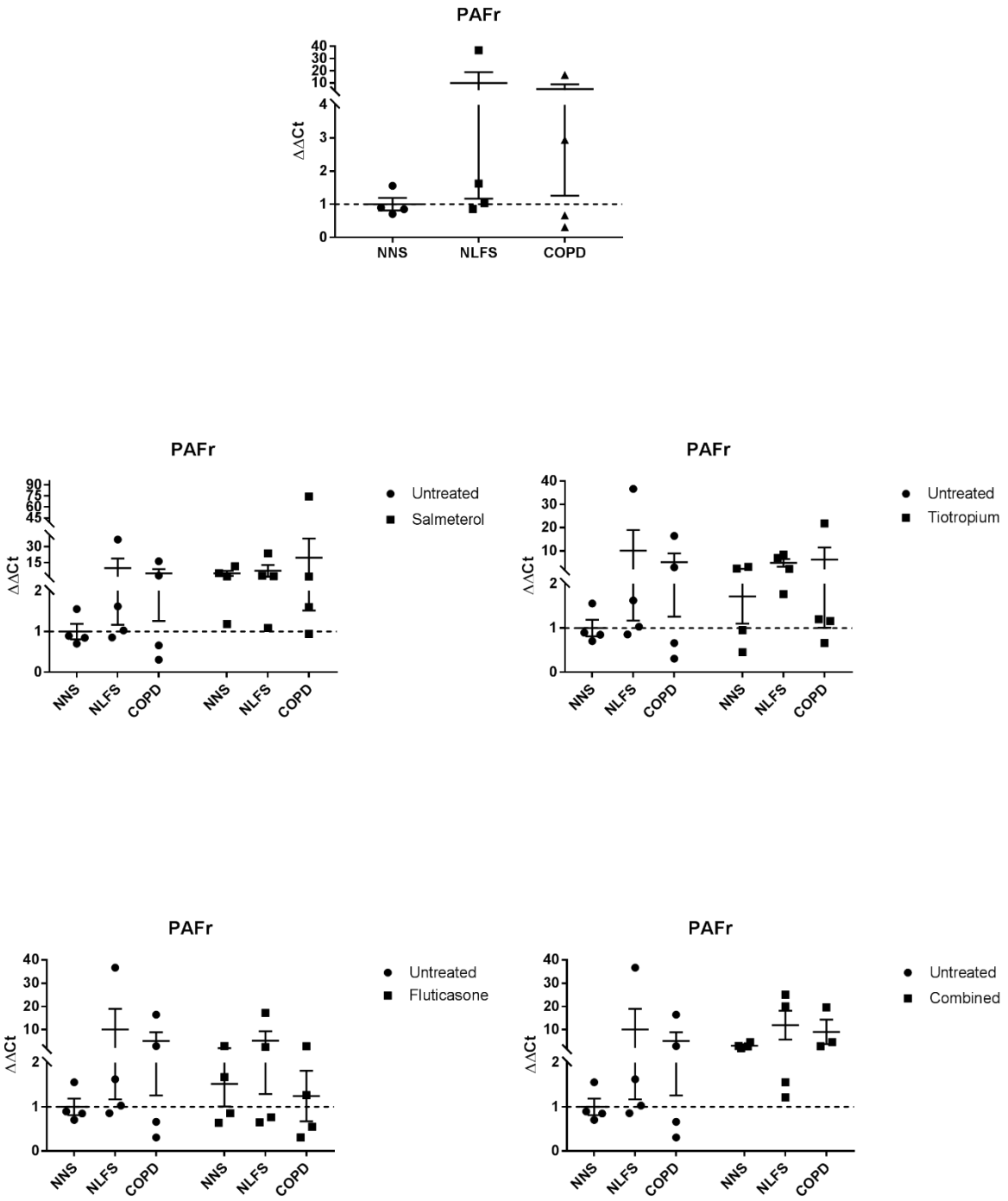

Figure 7.2-2: Comparison of bacterial adhesion molecule platelet-activating factor receptor (PAFr) mRNA expression in pHBECs derived from non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD)people with airflow limitation (COPD) treated with a LABA (salmeterol), LAMA (tiotropium) or corticosteroid (fluticasone). *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated non-smokers' cells. Data are expressed as mean with SEM. The dashed line indicates the expression of the untreated non-smokers' cells. ΔΔCT shows the fold-change in expression, with '1' being no change from baseline or the control. Each point on the graphs represents a single volunteer.*

Useful abbreviations

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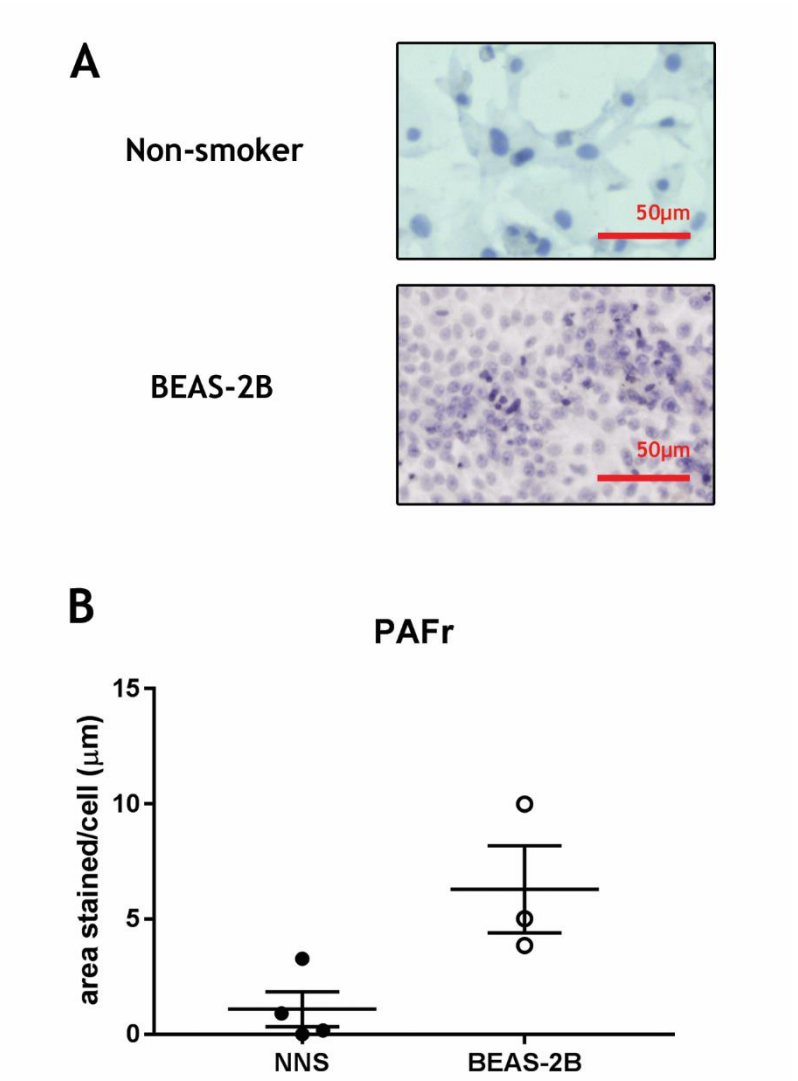

Figure 7.2-3: PAFr protein expression in non-smokers (NNS) compared to BEAS-2B cells as measured by immunocytochemistry. *Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer (NNS) or biological replicate (BEAS-2B).*

A: *Immunocytochemical images of non-smokers' cells and BEAS-2B cells. Cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin.* ***B:*** *Graphical representation of the area stained by immunocytochemistry. Data are represented as mean with SEM.*

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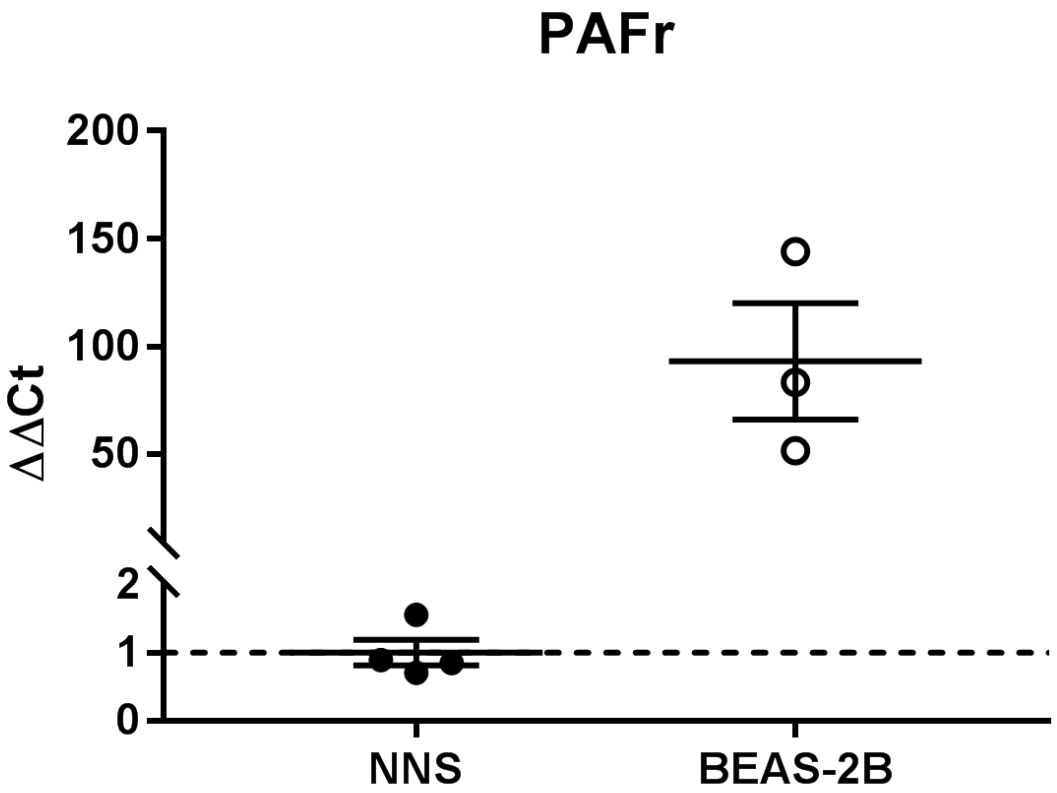

Figure 7.2-4. Comparison of bacterial adhesion molecule platelet-activating factor receptor (PAFr) mRNA expression in pHBECs derived from non-smokers (NNS) and the immortalised BEAS-2B cell line. *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated BEAS-2B cells. Data are expressed as mean with SEM. The dashed line indicates the expression of the untreated BEAS-2B cells. ΔΔCT shows the fold-change in expression, with '1' being no change from baseline or the control. Each point on the graphs represents a single volunteer or experimental sample.*

Useful abbreviations

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CSE *– cigarette smoke extract*

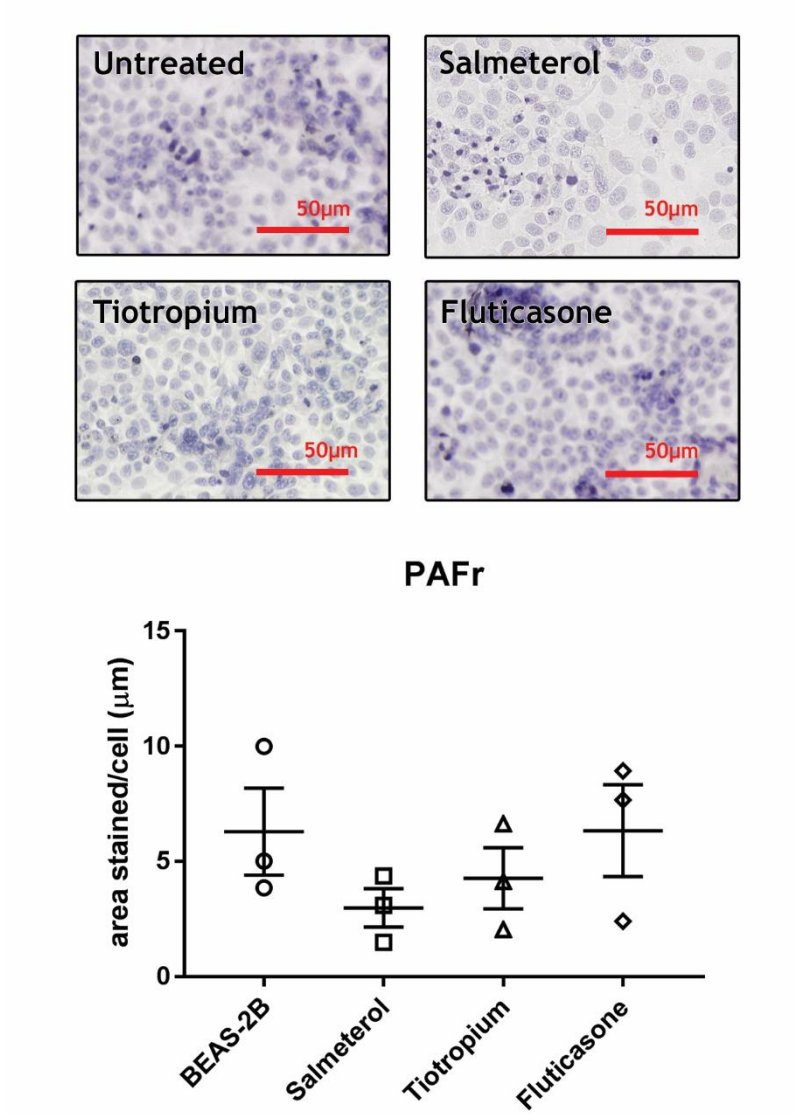

Figure 7.2-4: Platelet activating factor receptor (PAFr) protein expression in BEAS-2B cells with and without exposure to salmeterol, tiotropium or fluticasone for 24 hours as measured by immunocytochemistry. *Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single biological replicate.*

A: *Immunocytochemical images of BEAS-2B cells with and without drug treatment. Cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin.* ***B:*** *Graphical representation of the area stained by immunocytochemistry. Data are represented as mean with SEM.*

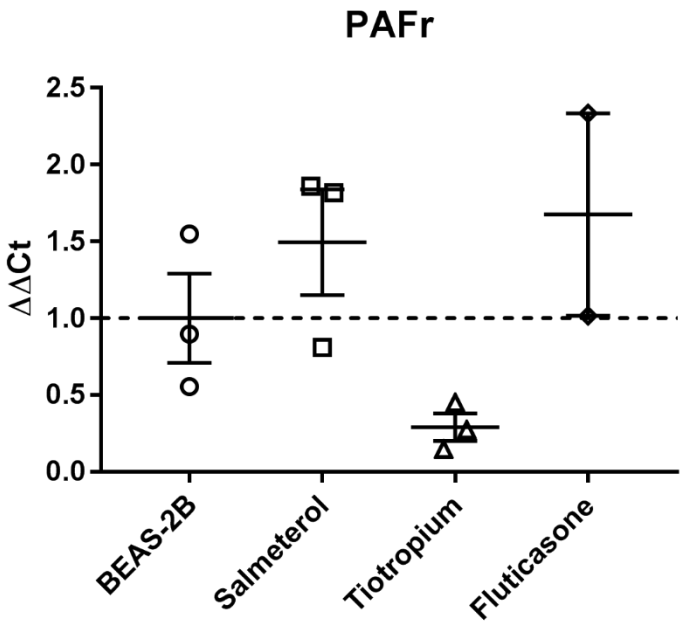

Figure 7.2-5: Comparison of bacterial adhesion molecule platelet-activating factor receptor (PAFr) mRNA expression in BEAS-2B cells following treatment with a LABA (salmeterol), LAMA (tiotropium) or corticosteroid (fluticasone). *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated BEAS-2B cells. Data are expressed as mean with SEM. The dashed line indicates the expression of the untreated BEAS-2B cells.*

7.2.1.2. PAFr expression in CSE exposed cells

In primary cells taken from non-smokers, exposure to cigarette smoke extract (CSE) for 4 hours increased PAFr protein expression by 22.2 times, although this did not reach statistical significance (Figure 7.2-7). The addition of salmeterol or fluticasone to the CSE treated cells did not affect PAFr protein expression, however combining CSE and tiotropium resulted in a statistically significant 51.7 times increase in PAFr protein expression (one-way ANOVA p = 0.0105). At the mRNA level, CSE exposure had no effect on PAFr expression (Figure 7.2-8), nor did any of the three drugs in combination with CSE affect PAFr mRNA expression.

While the BEAS-2B cells were similar to the pHBECs in that CSE had no effect on the mRNA expression of PAFr (Figure 7.2-9), BEASS-2B cells exhibited a 7.7-fold decrease in PAFr when treated with salmeterol and CSE (one-way ANOVA p = 0.0133), a 6.8-fold decrease when treated with tiotropium and CSE (one-way ANOVA p = 0.0149) and a 7.3-fold decrease following treatment with fluticasone and CSE (one-way ANOVA p = 0.0265) when compared to BEAS-2B cells treated with CSE alone (Figure 7.2-9). At the protein level, BEAS-2B cells exposed to CSE for 4 hours exhibited a decrease in PAFr expression 4.6 times (one-way ANOVA p = 0.0295) (Figure 7.2-10). The addition of salmeterol or fluticasone for 24 hours in addition to the CSE did not rescue this effect, and although tiotropium treated cells did not show a statistically significantly difference from the untreated cells, they appeared to be more similar to CSE treated cells than the control cells.

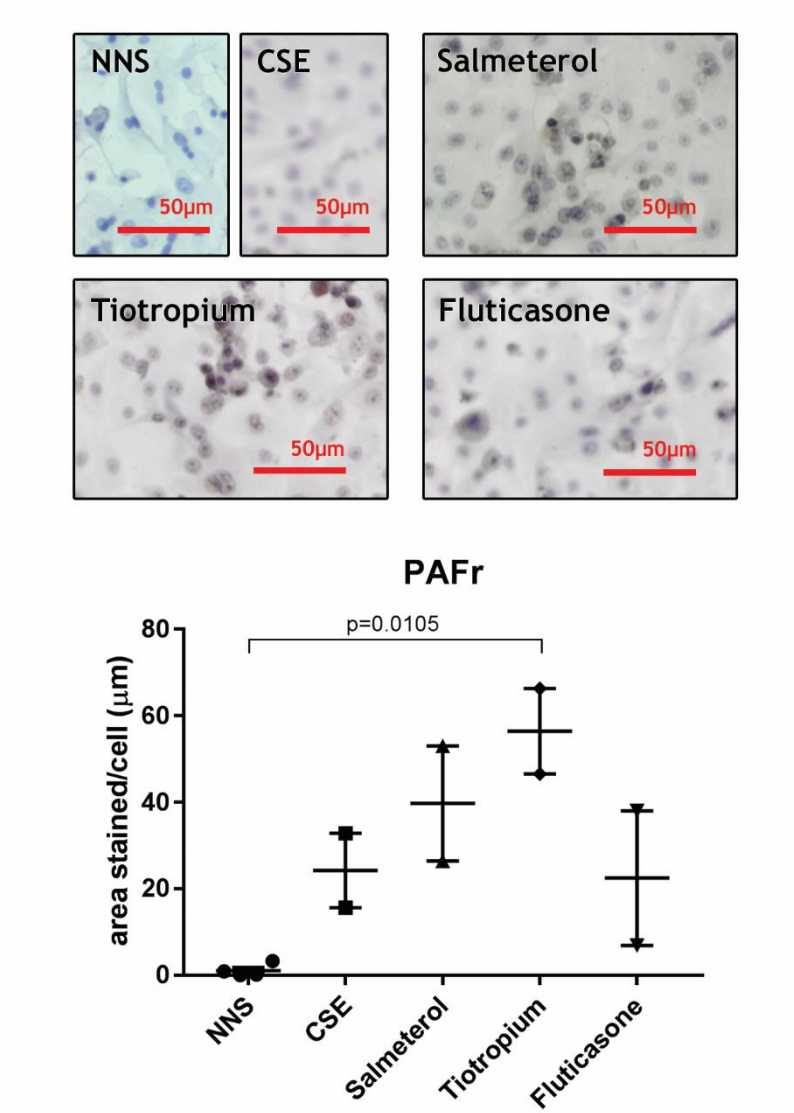

Figure 7.2-6: Platelet activating factor receptor (PAFr) protein expression in primary cells following exposure to salmeterol, tiotropium or fluticasone for 24 hours and cigarette smoke extract (CSE) for 4 hours, as measured by immunocytochemistry. *Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer.*

A: *Immunocytochemical images of non-smokers' cells treated with both CSE and the indicated drug. Cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin.* ***B:*** *Graphical representation of the area stained by immunocytochemistry. Data are represented as mean with SEM.*

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

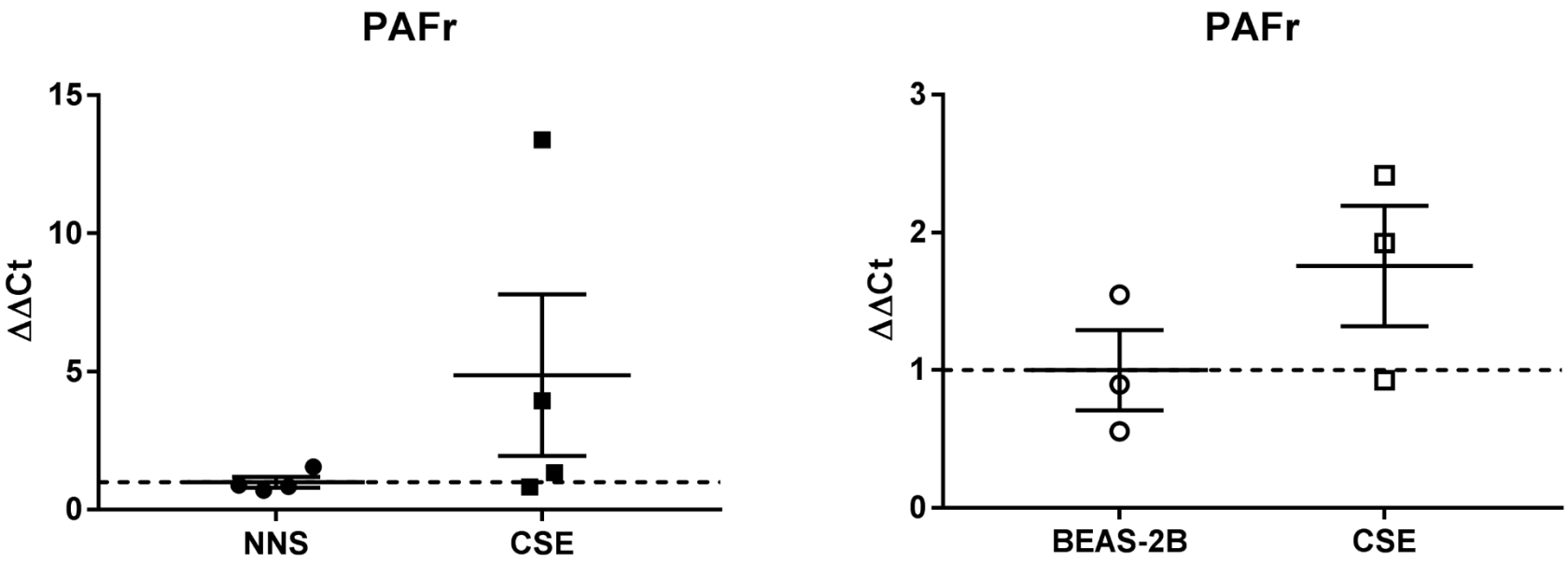

Figure 7.2-8. Comparison of bacterial adhesion molecule platelet-activating factor receptor (PAFr) mRNA expression in pHBECs derived from non-smokers (NNS) and the immortalised BEAS-2B cell line following treatment with CSEβ for 4 hours. *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. Data are expressed as mean with SEM. The dashed line indicates the expression of the untreated cells. ΔΔCT shows the fold-change in expression, with '1' being no change from baseline or the control. Each point on the graphs represents a single volunteer or experimental sample.*

Key: *Filled**Filled** shapes are NNS pHBECs, open shapes are BEAS-2B cells.*

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
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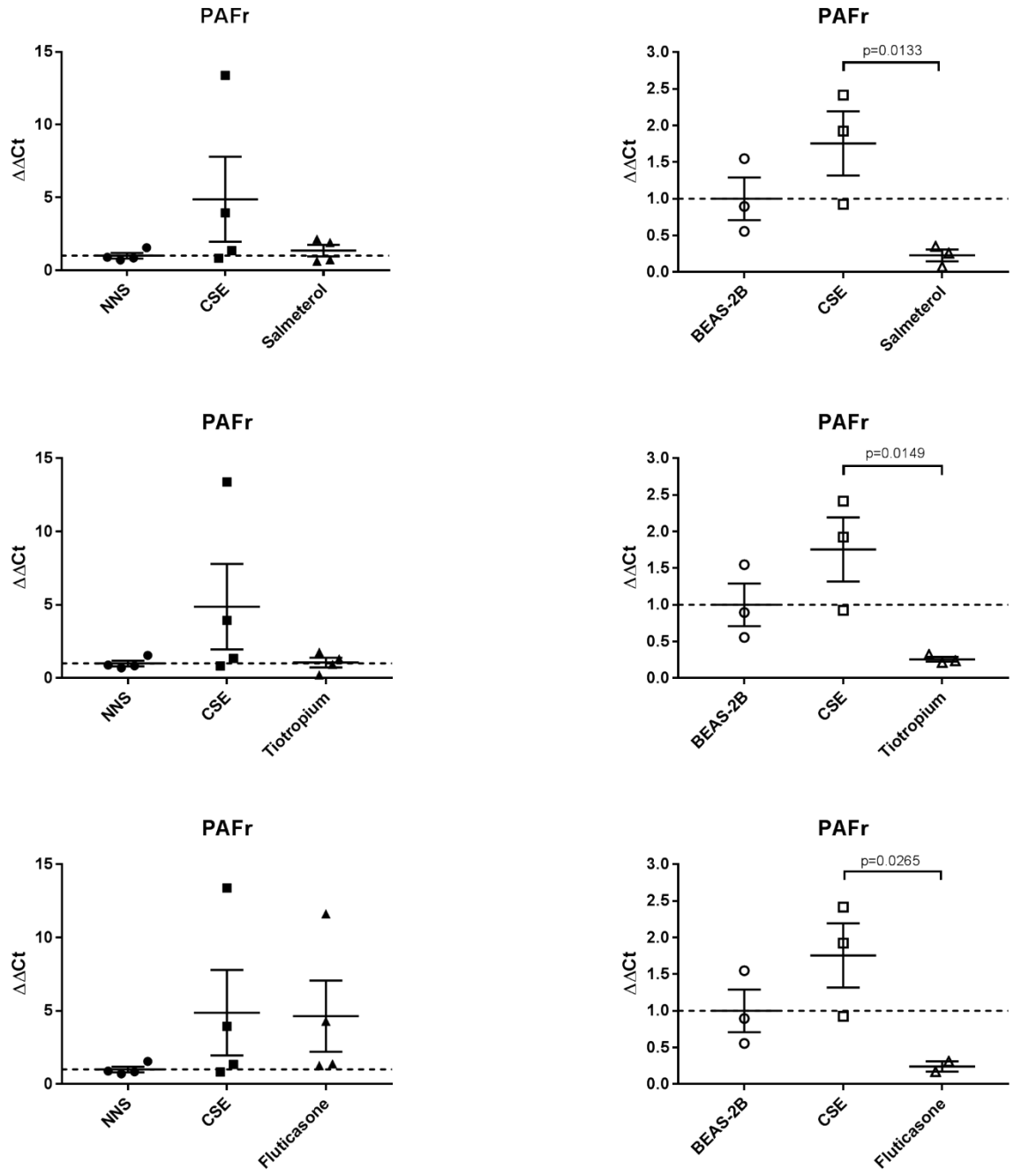

Figure 7.2-7: Comparison of bacterial adhesion molecule platelet-activating factor receptor (PAFr) mRNA expression in pHBECs derived from non-smokers (NNS) and the immortalised BEAS-2B cell line following treatment with CSE for 4 hours and exposure to a LABA (salmeterol), LAMA (tiotropium) or corticosteroid (fluticasone) for 24 hours. *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. Data are expressed as mean with SEM. The dashed line indicates the expression of the untreated cells.*

Key: *Coloured shapesFilled shapes are NNS pHBECs, open shapes are BEAS-2B cells.*

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
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LABA/LAMA – *long-acting β-agonist/muscarinic antagonist*
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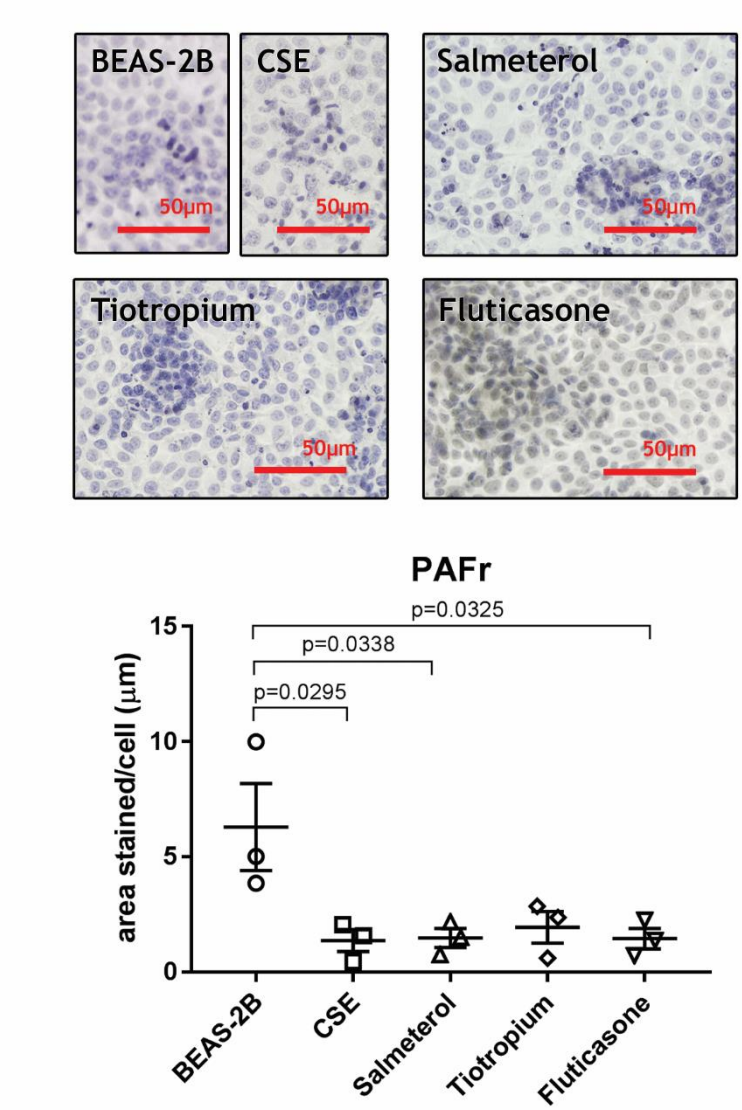

Figure 7.2-8: Platelet activating factor receptor (PAFr) protein expression in BEAS-2B cells following exposure to salmeterol, tiotropium or fluticasone for 24 hours and cigarette smoke extract (CSE) for 4 hours, as measured by immunocytochemistry. *Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single biological replicate.*

A: *Immunocytochemical images of BEAS-2B cells treated with both CSE and the indicated drug. Cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin.* ***B:*** *Graphical representation of the area stained by immunocytochemistry. Data are represented as mean with SEM.*

Useful abbreviations

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7.2.1.3. PAFr expression in TGF-β exposed cells

Exposure of primary cells from non-smokers to transforming growth factor β1 (TGF-β) appeared to increase PAFr expression at the protein level by 22.9 times, however this did not reach statistical significance (Figure 7.2-11). Cells exposed to a combination of TGF-β with salmeterol, tiotropium or fluticasone were not significantly different from baseline nor cells treated with TGF-β alone, although they appeared to more closely resemble cells exposed to TGF-β than unstimulated cellscells.

In BEAS-2B cells TGF-β, alone or in combination with salmeterol, tiotropium or fluticasone, had no effect on PAFr protein levels (Figure 7.2-12). At the mRNA level, TGF-β either with or without drugs did not alter PAFr expression in either primary or BEAS-2B cells (Figure 7.2-13).

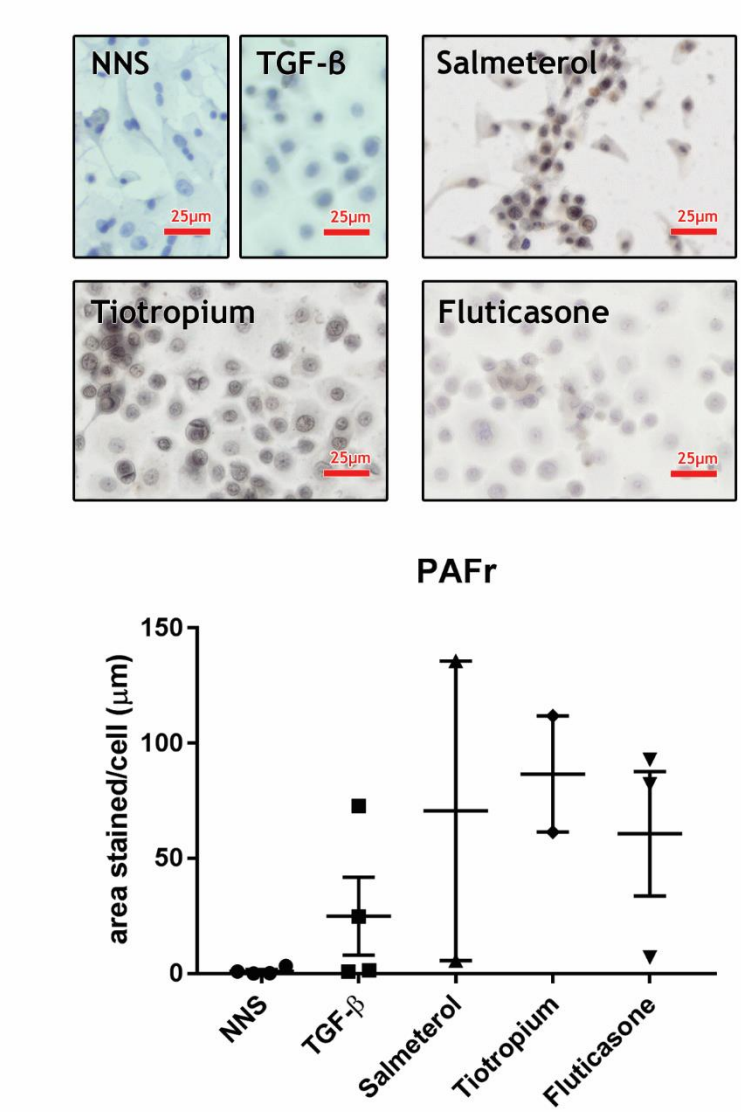

Figure 7.2-9: Platelet activating factor receptor (PAFr) protein expression in primary cells following exposure to transforming growth factor β1 (TGF-β) for 72 hours and salmeterol, tiotropium or fluticasone for 24 hours as measured by immunocytochemistry. *Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer.*

A: *Immunocytochemical images of non-smokers' cells treated with both TGF-β and the indicated drug. Cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin.* ***B:*** *Graphical representation of the area stained by immunocytochemistry. Data are represented as mean with SEM.*

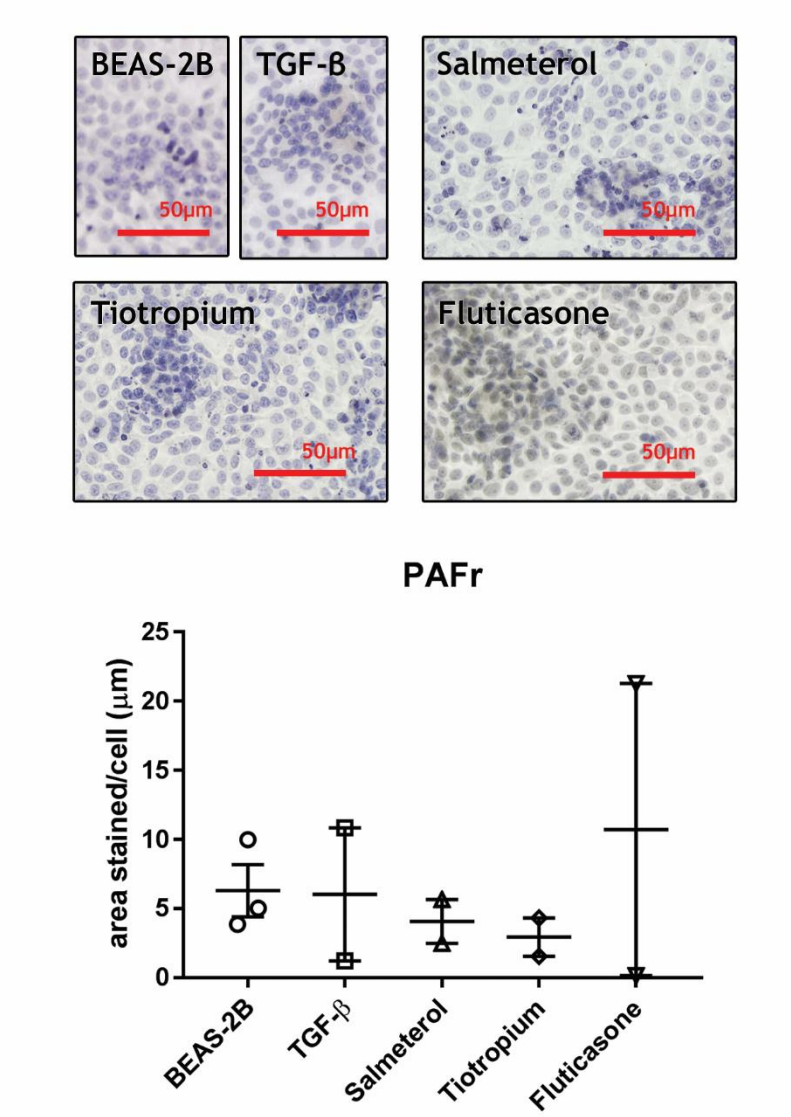

Figure 7.2-10: Platelet activating factor receptor (PAFr) protein expression in BEAS-2B cells following exposure to transforming growth factor β1 (TGF-β) for 72 hours and salmeterol, tiotropium or fluticasone for 24 hours as measured by immunocytochemistry. *Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single biological replicate.*

A: *Immunocytochemical images of BEAS-2B cells treated with both TGF-β and the indicated drug. Cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin.* ***B:*** *Graphical representation of the area stained by immunocytochemistry. Data are represented as mean with SEM.*

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting ß-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-ß *– transforming growth factor-ß1*
CSE *– cigarette smoke extract*

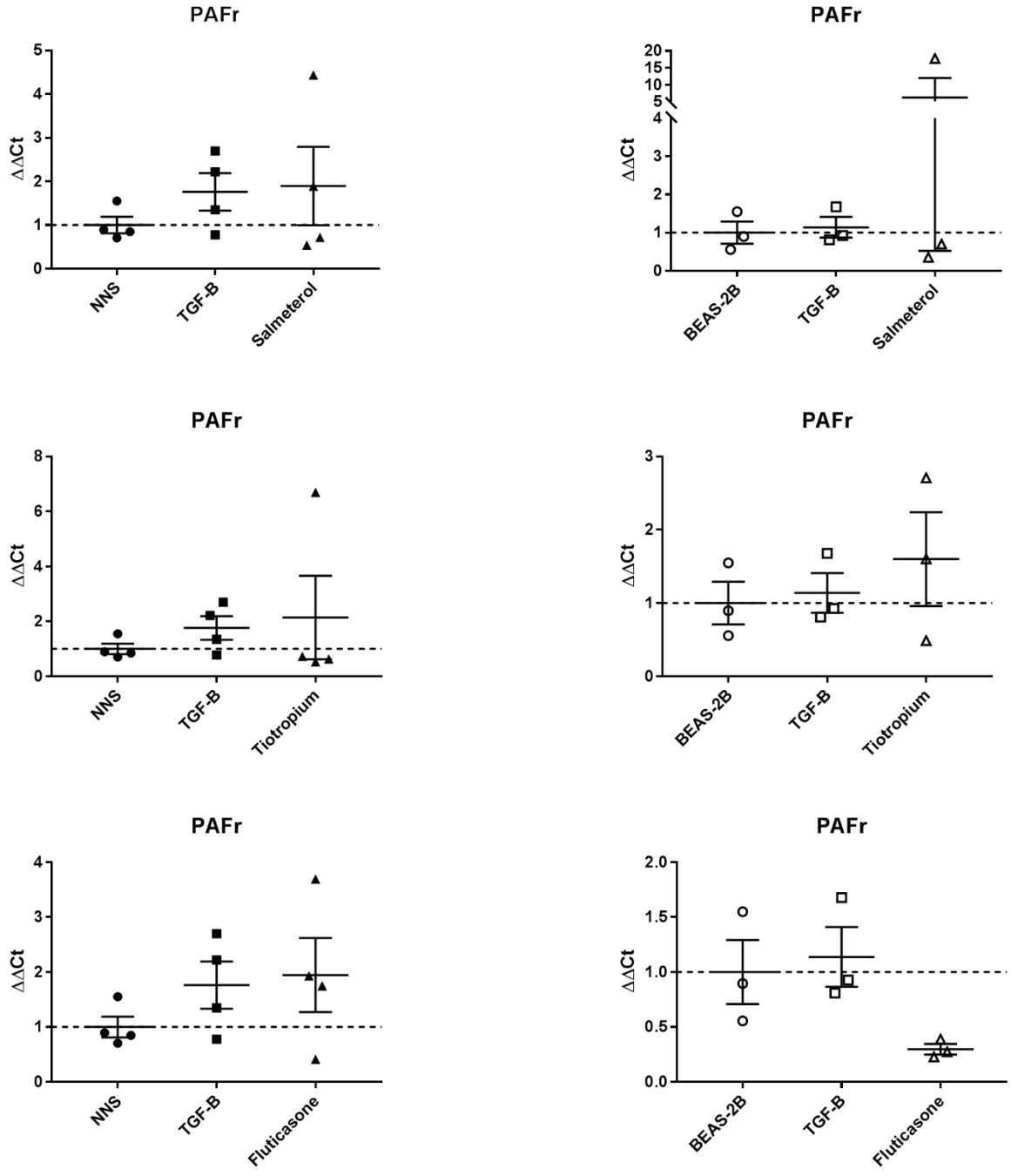

Figure 7.2-13. Comparison of bacterial adhesion molecule platelet-activating factor receptor (PAFr) mRNA expression in pHBECs derived from non-smokers (NNS) and the immortalised BEAS-2B cell line following treatment with TGF-β for 72 hours and exposure to a LABA (salmeterol), LAMA (tiotropium) or corticosteroid (fluticasone) for 24 hours. *Gene expression was normalised to β-actin as the housekeeping gene, and further normalised to the average expression in untreated cells. Data are expressed as mean with SEM. The dashed line indicates the expression of the untreated cells. ΔΔCT shows the fold-change in expression, with '1' being no change from baseline or the control. Each point on the graphs represents a single volunteer or experimental sample.*
Key: *FilledFilled shapes are NNS pHBECs, open shapes are BEAS-2B cells.*

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
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pHBECs *– primary human bronchial epithelial cells*
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7.2.2. ICAM-1

7.2.2.1. ICAM-1 expression in non-smokers, smokers with normal lung function and people with airflow limitation

There was no significant difference in ICAM-1 protein expression across cells taken from non-smokers, smokers with normal lung function and people with airflow limitation (Figure 7.2-14), although there was a suggestion that smokers with normal lung function may have two distinct groups, one with low ICAM-1 expression and one with high ICAM-1 expression.

There appeared to be no effect in any of the three groups on ICAM-1 expression at the protein level by exposure to salmeterol (Figure 7.2-14) , tiotropium (Figure 7.2-14) , fluticasone (Figure 7.2-14) or all three drugs combined (Figure 7.2-14). ICAM-1 levels appeared unaffected by exposure to salmeterol, tiotropium or fluticasone and cells from smokers or people with airflow limitation did not express levels of ICAM-1 which differed from non-smokers, although there may be a sub-group of smokers with normal lung function which express it highly.

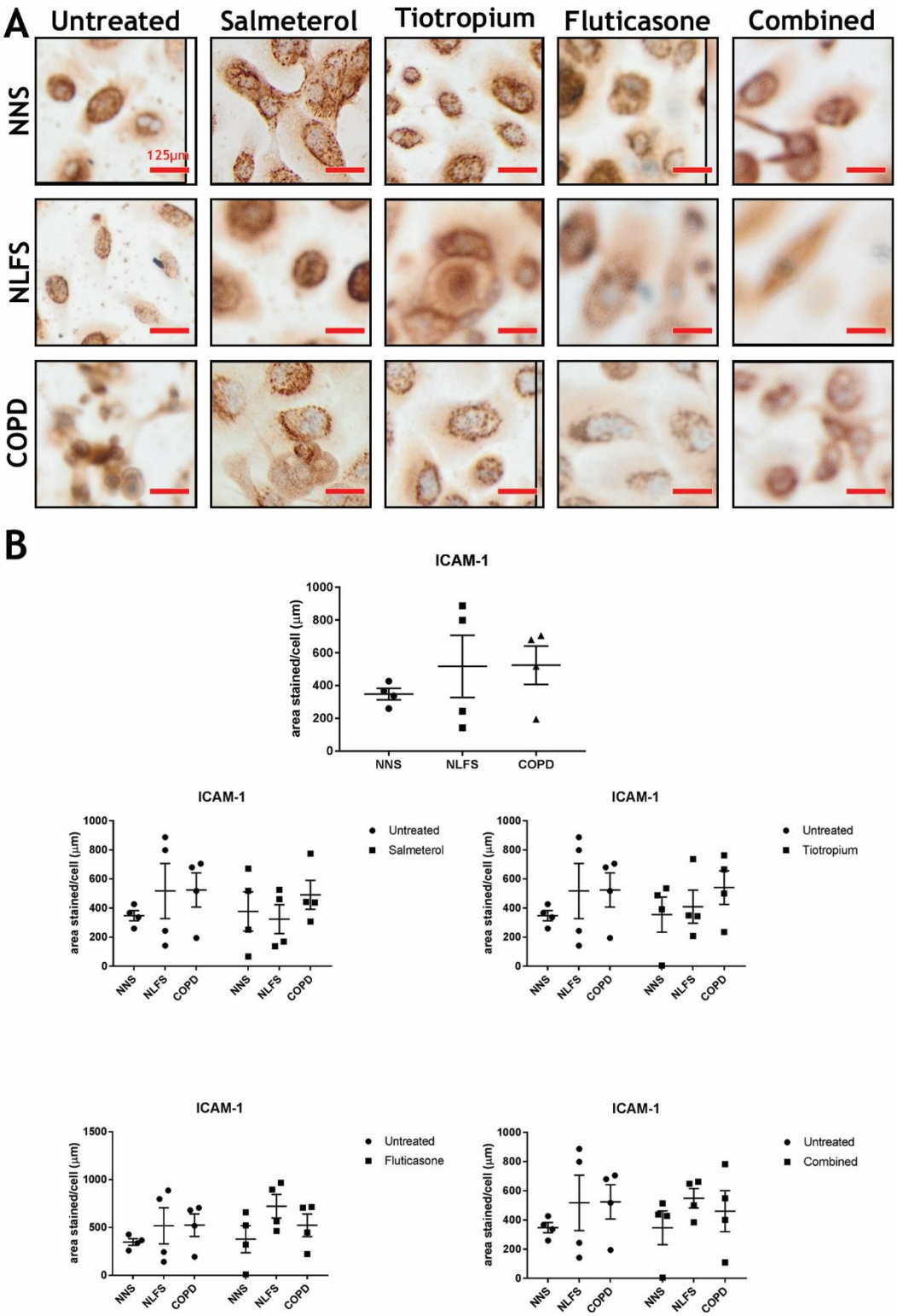

Figure 7.2-11: Baseline ICAM-1 protein expression in non-smokers (NNS), smokers with normal lung function (NLFS) and people with COPD (COPD) as measured by immunocytochemistry with and without exposure to drugs. *Each sample measured had five non-overlapping fields of view measured individually, and the results were averaged to get a single final value for each sample. Each point on the graphs represents a single volunteer.*

A: *Immunocytochemical images of cells from non-smokers, smokers with normal lung function and people with airflow limitation (COPD). Cellular protein (brown) was stained with DAB. Nuclei (blue) were stained with Meyer's haematoxylin.* ***B:*** *Graphical representation of the area stained by immunocytochemistry quantified in the three groups. Data are represented as mean with SEM.*

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

7.3. Chapter discussion

7.3.1. PAFr

In this study, there did not appear to be any difference in expression of the bacterial adhesion factor PAFr between cells from non-smokers, smokers with normal lung function and people with airflow limitation. Salmeterol, tiotropium or fluticasone individually appeared to have no effects on PAFr expression, although the combination of the three drugs significantly increased PAFr expression in cells from non-smokers. Although interesting, this information does not have any real clinicalclinical significance, as healthy individuals would not be prescribed these drugs.

This study agreed with the literature [177, 199], demonstrating a trend towards PAFr upregulation in primary cells following exposure to cigarette smoke extract (CSE). This study further demonstrated that this effect was exacerbated by exposure to tiotropium. This result suggests that tiotropium may not be a preferable method of treatment for airflow obstruction or COPD in people who continue to smoke, although it would likely be acceptable for ex-smokers.

TGF-β had no effect on PAFr expression in this study, although when combined with the three drugs there was a trend towards increased expression, suggesting that there may be some activity. Further work utilising fresh TGF-β is required to confirm this trend.

BEAS-2B cells, in contrast to the primary cells and previous literature nono change in expression of PAFr following exposure to CSE, although it was interesting to note that BEAS-2B cells exposed to CSE and any of salmeterol, tiotropium or fluticasone exhibited a significant decrease in PAFr expression compared to cells exposed to CSE alone.

Furthermore, TGF-β exposure had no effect on PAFr expression, nor did any of the three drugs appear to affect PAFr expression when combined with TGF-β. These results once again demonstrated that the BEAS-2B cell line is an unreliable and generally inaccurate model of healthy primary bronchial epithelium, and that care should be taken if utilising it to test drug therapies.

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
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COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

7.3.2. ICAM-1

This study demonstrated that ICAM-1 did not appear to be upregulated in the large airways of current smokers or people with airflow limitation compared to healthy non-smokers. Previous literature has shown that soluble ICAM-1 is elevated in COPD [243, 247-251], however the majority of work looking at ICAM-1 expression in COPD has been focussed on ICAM-1 expression in bronchoalveolar lavage fluid (BALF) or serum [243, 248-251] with only a limited number of examinations of the airway wall and epithelium [247]. This may have been partiallyresponsible for the differences in ICAM-1 expression observed in this study, however it is unlikely the whole story, since it is known to be upregulated in the epithelial layer [247].

This study was limited by small sample sizes, however the most likely explanation for the lack of change in ICAM-1 expression in cells from people with airflow limitation is that increased ICAM-1 is present in more developed COPD, but not in early stages of airflow limitation. Within this study, there was no evidence that salmeterol, tiotropium or fluticasone had any effect on ICAM-1 expression, either alone or in combination. However, further study of the drug effects in either artificially activated epithelium or in epithelium derived from clinical COPD may show different results, and is highly recommended. Additionally, it would be advisable to compare cultured cells to biospies, in order to confirm that culture conditions have not adversely affected ICAM-1 expression, and perhaps erased changes in the diseased cells.

7.4. Chapter conclusions

Neither PAFr nor ICAM-1 appeared to be upregulated in cells from people with chronic airflow limitation. Similarly, PAFr and ICAM-1 expression was not affected by salmeterol, tiotropium or fluticasone. While a combination of the three drugs did cause increased expression of PAFr, this effect was only present in cells taken from non-smokers and the drug cocktail had no effect on ICAM-1 expression in any group. The clinical relevance of this result is unlikely to be important, however it was also seen that tiotropium and cigarette smoke extract together significantly increased PAFr expression, suggesting that tiotropium may not be an ideal treatment choice for current smokers. As in previous sections of this study, the small number of samples limited the statistical analysis of these data, however the results were useful for indicating possible areas for further investigation.

The BEAS-2B cell line, which is commonly utilised in research when primary human bronchial epithelial cells (pHBECs) are unavailable, is, as in previous chapters, bears only a slight relevance to true healthy pHBECs when observing PAFr expression. Although not sufficiently powered to detect statistical significance, it appeared that the BEAS-2B cells expressed higher levels of PAFr at baseline than primary cells from non-smokers. Furthermore, the BEAS-2B cells appeared to react differently to CSE than primary cells. Although the results did not reach statistical significance, there appeared to be upregulation of PAFr in primary cells exposed to CSE, while BEAS-2B cells exhibited a decrease in expression. It is essential that further study on this topic is undertaken, as it suggests that BEAS-2B cells are not only a less than ideal model for EMT but may have major flaws as a model for activated airway epithelium in general.

8. General discussion

This thesis aimed to determine whether epithelial mesenchymal transition (EMT), a process which is known to be active in chronic obstructive pulmonary disease (COPD) [31-34], is active at early stages of the disease. Primary cells were isolated from non-smokers, smokers with normal lung function and people with airflow obstruction which did not reach the cut-off for a clinical diagnosis of COPD [208]. In this study iti appeared that, although EMT is thought to play a role in established COPD [31-34], at very early stages of airflow obstruction EMT diddid not appearappeare to be active.

There still remains the question of what triggers the process of EMT in COPD - cigarette smoke has been shown to induce EMT in bronchial epithelial cells from smokers, as well as in non-smokers with lung cancer [33, 238, 307, 308]. It has been demonstrated to act, at least partially, via the TGF-β signalling pathway [33, 233, 265], and TGF-β *in vitro* is known to be a potent inducer of EMT in epithelial cells derived from a variety of organs [58, 65, 79-82]. This CSE-TGF-β-EMT pathway has been demonstrated in primary bronchial epithelial cells (pHBECs) from non-smokers who have lung cancer [33], and TGF-β alone activates EMT in both pHBECs and the immortalised cell line BEAS-2B [165, 207, 257]. However, the response of healthy pHBECs to cigarette smoke required further investigation, in order to study what effects the initiation of smoking might have on a person's airways.

In this study, exposure to cigarette smoke extract (CSE) did not induce complete EMT in primary cells from non-smokers, despite the evidence of previous literature [33]. TheT mesenchymal marker N-cadherin was significantly increased in response to CSE, however CSE exposure significantly reduced expression of fibrotic protein collagen 1-α. It was likely,

however, that some, if not all, of this unexpected decrease in pro-collagen 1-α expression was due to the short amount of time the cells were exposed to the CSE-containing media, which would have resulted in less accumulation of secreted factors and proteins than the control media, which was applied to the cells for longer prior to collection. CSE also did not affect expression of Smad6, a molecule involved in the TGF-β pathway. Based on these data, it would appear that while acute exposure to CSE did affect healthy pHBECs, they wereare somewhat resistant to induction of complete EMT. Further, TGF-β did not appear to be involved in the induction of the EMT-like effect, at least not through the Smad pathway. It is possible that the MAPK/Erk pathway may have been involved, as it has been implicated in TGF-β induced EMT in the literature [92].

The response of both primary and BEAS-2B cells to TGF-β in this study appeared to be lower than expected when compared with previous literature [165, 257], however it appeared to produceproduce some indications of a mild, EMT-like phenotype in the BEAS-2B cells, although it did not affect all markers studied. This would suggest that although the changes seen in this study were slight, they may be real effects partly obscured by a dulled response and are worth further investigation. Especially important is confirmation ofthe fact that the biological activity of TGF-β should ideally be confirmed before attempting work, possibly utilising the MBF-11 or TMLC cell line-based bioassayslines [267, 268].

All three drugs examined as part of this study (salmeterol, tiotropium and fluticasone) appeared havehave no effect on either TWIST or Smad6 signalling, which is unexpectedun given previous literature on salmeterol [277] and tiotropium [289].. All three drugs are known to have effects on the epithelial layer of the airways [172, 272, 282, 295, 296], however this is the first time their effect on EMT has been directly measured. Salmeterol has

previously been shown to reduce epithelial wound healing and repair [272], a process which is directly linked to EMT, however there has not been a molecular-level examination of EMT following exposure to the drug. In this study, salmeterol had no significant effect on EMT-related markers.

On the contrary, tiotropium exposure in cells from people without airway obstruction upregulated some markers of EMT, although most of this upregulation was limited to the mRNA level, without associated protein level changes. Of special interest was the fact that tiotropium, when combined with CSE, appeared to increase expression of the mesenchymal marker vimentin in primary and N-cadherin in BEAS-2B cells. This promotion of mesenchymal marker expression suggests that tiotropium may not be an ideal treatment for people with COPD who continue to smoke.

In addition to the primary aims, this study also allowed collection of valuable technical information. It showed that pHBECs cells can be successfully stored using cryopreservation, although the current smoking status of the cells' donor negatively affected the viability of the cells following storage. Airflow obstruction, however, appeared to have no effect on cell growth or viability during cryopreservation. This is a novel finding, as within the literature pHBECs are used without cryopreservation [33, 103, 111, 192, 194, 231-236]. It indicates that pHBECs can be stored for future use, although it would be recommended to attempt optimisation of the recovery process to aid survival of cells taken from current smokers.

Furthermore, this study directly compared pHBECs from healthy non-smokers to the immortalised BEAS-2B cell line. The immortalised BEAS-2B cells, despite its prevalence as a model of healthy bronchial epithelial cells, were demonstrated to be substantially more mesenchymal when compared to pHBECs, as well as expressing higher levels of PAFr.

These differences were observable both at baseline and in the cells' responses to stimulus and drug treatments. However, the differences appeared to be restricted primarily to the molecular level rather than the protein level expression. This suggests that if utilising the BEAS-2B cell line to model healthy bronchial epithelial cells, it is preferable to study protein-level changes as compared to mRNA level changes.

This study comprised of a collection of primary cells from volunteers with well-phenotyped clinical airway characteristics. However, due to the logistics of collection and the effects of cryopreservation, not to mention the limited number of samples collected in the smoking and disease groups and limited available time for experimental procedures and analysis, the number of samples able to be utilised for this study was low.

Due to the limited number of samples, this study focussed more on a broad preliminary approach, examining a number of markers of EMT and selected associated pathways. While it would have been better from a statistical standpoint to look at a larger number of samples, possibly sacrificing some markers, it was not feasible within this study. As such, the results of this study should be considered to suggest future areas requiring more in-depth study rather than as conclusive answers in their own right.

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

8.1. Future directions

Although the results of these studies did not, overall, yield any statistically significant novel findings, they did provide some potentially interesting insights into EMT in airflow limitation, as well as information pertinent to both primary and immortalised bronchial epithelial cell culture. These results provide a rationale for should be explored in future work directedin order to confirmingfirmly establish or deny the patterns observed inseen these studies, and can provide a basis for further study.

The most potentially interesting, and concerning, result of these studies was the observation that primary bronchial epithelial cells from non-smokers and the immortal BEAS-2B cell line not only exhibited baseline differences in their expression of EMT-related markers but also appeared to respond differently to both inducers such as TGF-β and CSE as well as to the different drug therapies. As previously discussed (section (1.3.1.2), the BEAS-2B cell line is both known and expected to differ at baseline from primary cells in a number of ways, including its inability to differentiate when cultured at an air-liquid interface. However, for experimental purposessince it is widely considered to be representative of 'normal', healthy bronchial epithelium, and thus needsit would be expected to behave in a similar fashion to primary bronchial epithelialepithleial cells from healthy individualsindivduals when exposed to stimuli. The results of the studies undertaken within this thesis suggest that the BEAS-2B cell line may not, in fact, be an ideal model for normal bronchial epithelial cells and further work should be undertaken to either confirm or deny this, not only in BEAS-2B cells but perhaps also in other commercially available cells such as the 16HBE cell line.. A more detailed examination of signalling pathways, as well as an increased number of primary cell samples would be an excellent starting point, as would synchronising cell cycles prior to

extraction of mRNA and protein work in order to reduce variation between samples. Overall, the possibility that the BEAS-2B cell line has a more mesenchymal phenotype than normal bronchial epithelium at baseline means that these cells may not be ideal for studies of EMT, wound healing and cell migration, or other studies that rely on the cells exhibiting a healthy epithelial profile.

In a similar vein, although the current studies showed no difference in EMT markers between cells taken from non-smokers and people with mild, non-COPD airflow limitation, the inclusion of cells taken from people with clinically-defined COPD, especially Stage II or III, would be of enormous benefit, since it could shed light on the role of EMT in not only mild airflow limitation but in established obstructive pulmonary disease. Furthermore, additional experimental techniques such as RNAScope in biopsy samples and Western blotting in cultured cell samples, as well as examining cells differentiated at an air-liquid interface could provide a more complete picture of the changes occurring in airflow obstruction, both pre-clinically and in COPD. Although attempts were made to apply RNAScope to the current studies, the way the samples had been processed and stored proved incompatible with the available techniques, however future studies can ensure that sample collection and preservation are suited for these technologies.

The need forFurther study of the effects of smoking on primary cell survival and growth were also highly suggested by the results of these studies. Smoking, more than airflow limitation, appeared to play a key role in determining cell survival, especially following cryopreservation. The current studies were not equipped to investigate this result in any depth, and further study of the mechanism behind this phenomenonphenomenom is warranted, possibly examining. This study was not designed or equipped to investigate this

interesting result, however it may be of importance in understanding the DAMP/more acute effects of smoking, and quitting, on the airway epithelium. It is possible that the Nrf-2 and DAMP signalling pathways may be involved, as they have been implicated in cigarette smoke induced apoptosis and cell death in the airway epithelium in a number of studies [309-311]

Overall, despite being underpowered to make strong statistically supported claims, the studies in this thesis have highlighted a number of potentially interesting areas where further research could dramatically enhance not only our understanding of EMT and airflow limitation, but also reveal potentially important and previously under-recognisedunderrecognised differences between immortalised cell lines and healthy cells.

Useful abbreviations

NNS *– non-smokers*
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COPD-ES *– ex-smokers with airflow limitation*
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pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

9. General conclusions

The results of this study indicated that while healthy bronchial epithelial cells are negatively affected by exposure to cigarette smoke, undergoing partial EMT and showing signs of a trend towards upregulation of bacterial adhesion site PAFr, the cigarette smoke extract-transforming growth factor-β1-epithelial-mesenchymal transition (CSE-TGF-β-EMT) pathway may not be the initial mechanism behind the damage caused by cigarette smoke. This suggestion is further advanced by the fact that TGF-β induced EMT appeared to exhibit a different expression profile from that induced by CSE, although in this study the changes induced by TGF-β did not reach statistical significance.

This study also demonstrated that in pre-clinical COPD, which is to say in people with airflow obstruction which does not reach the threshold for a clinical diagnosis of COPD, EMT did not appear to be active in the airway epithelium. This provides further corroboration for the conclusion that EMT is not continuously active in the epithelium until the disease is fully established. Furthermore, while the long-acting β-agonist salmeterol and the long-acting muscarinic antagonist may act to reduce EMT caused by cigarette smoke extract, they have no benefit when applied to cells which are not activated, nor do they appear to act by affecting TGF-β signalling. Tiotropium may even be mildly detrimental in people without airflow obstruction, and particularly when combined with cigarette smoke, suggesting that care should be taken when considering prescribing these drugs for treatment of early COPD.

This study also demonstrated that the BEAS-2B cell line, which is commonly used as a model for healthy bronchial epithelial cells not only is more mesenchymal at baseline than cells taken from non-smokers but also responds differently to stimuli. This was especially

true at the mRNA level, and raises questions regarding the suitability of this cell line as a model for healthy tissue.

However, the conclusions drawn from this study were hampered by a lack of power and were indicated by apparent trends rather than defined, clear signals. Further work is required to ascertain if these trends represented true and consistent signals. Despite this drawback, this study provided evidence that prolonged EMT activity not be an early, pre-COPD phenomena, and that late-stage therapies such as tiotropium may be detrimental if applied at early disease stages or in patients whom do not cease smoking.

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pHBECs *– primary human bronchial epithelial cells*
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CSE *– cigarette smoke extract*

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CSE *– cigarette smoke extract*

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Appendix 1: FIJI and ImageJ Macros (Raw Code)

Macro for automatically converting .vsi files into .tiff file format using FIJI

The function of this macro was to iterate through a folder containing multiple files saved from the Olympus Virtual Slide Scanner VS120 and convert them into .tiff files using the BioFormats plugin. The macro was designed to go through every file within a folder.

Functionality was built in to allow the user to alter which file format the macro would open and which format it would convert to, as well as allowing the user to change the resolution of the image using the 'series' which are a feature of the .vsi file format. Coding language is IJ1/FIJI native language.

```
Dialog.create("Start up");

Dialog.addMessage("Hi there! Hopefully this will work!");

Dialog.show();



//ZChoice = "Yes"



//Choose starting filetype

Dialog.create("File Type");

Dialog.addString("What type of file are you processing?",".vsi",5);

Dialog.show();

filetype = Dialog.getString();



//Choose export/save filetype

Dialog.create("Convert to?");

Dialog.addString("What file type would you like to convert \n the files into (no dots

please)?","tiff",5);

Dialog.show();

convert = Dialog.getString();
```

```
//Step completed visual confirmation

print(filetype+" files will be converted to ."+convert+" files.");



//Eventually become useful if multiple Z-planes needed to be imaged

//v= newArray()



//do {

//Dialog.create("Z-Planes")

//Dialog.addNumber("Which Z-Plane would you like to use?", 1)

//Dialog.show();



//z = Dialog.getNumber;



//v = Array.concat(v, z);

//Array.sort(v);

//z = Array.copy(v);

//Array.print(v);



//Dialog.create("Additional Z-Planes")
```

```
//Dialog.addChoice("Would you like to add another Z-Plane?", newArray("No", "Yes"))

//Dialog.show();

//ZChoice = Dialog.getChoice;



//} while (ZChoice == "Yes");



//Choose 'series' (resolution) that bioformats exports

Dialog.create("Series choice");

Dialog.addMessage("Which series would you like to convert? (I recommend series 2 for
general use.)");

Dialog.addCheckboxGroup(3,3,newArray(1,2,3,4,5,6,7),newArray(false,true,false,false,false,
false,false));

Dialog.show();

series1= Dialog.getCheckbox();

series2= Dialog.getCheckbox();

series3= Dialog.getCheckbox();

series4= Dialog.getCheckbox();

series5= Dialog.getCheckbox();

series6= Dialog.getCheckbox();
```

```
series7= Dialog.getCheckbox();


//Defines if multiple series chosen

seriesnum=series1+series2+series3+series4+series5+series6+series7


//List of series selected

if(series1==1){String.append(" Series_1")};if(series2==1){String.append("
Series_2")};if(series3==1){String.append(" Series_3")};if(series4==1){String.append("
Series_4")};if(series5==1){String.append(" Series_5")};if(series6==1){String.append("
Series_6")};if(series7==1){String.append(" Series_7")};

seriesstring=String.buffer;

print("Series to be exported: "+seriesstring);


//Eventually to be functional if only want to select files not whole folder

Dialog.create("Import select files or the whole folder?")

Dialog.addChoice("Files or Folder?", newArray("Folder", "Files"))

Dialog.show();

Input=Dialog.getChoice;
```

```
//Simple selection of folder with files and folder to save in
if(Input=="Folder"){
	inDir= getDirectory("Choose the folder to open.");
	outDir= getDirectory("Choose where to save the files.");
};

//Placeholder while 'file' option is broken
if(Input=="Files"){
	Dialog.create("File location information.");
	Dialog.addMessage("Please note that the files you wish to convert must be in the same folder, and they must be in an unbroken sequence. Unfortunately, I can't figure out how to make this program select only specific files, so it relies on file location within the folder. Sorry about that. :)");
	Dialog.show();
	Dialog.create("Enter the file range you wish to convert.")
	Dialog.addString("Please enter the number of the first file you wish to convert - count from the first file in the folder, ignoring any subfolders.","# here",5);
	Dialog.show();
	start=Dialog.getString();
```

```
	Dialog.create("Enter the file range you wish to convert.")

	Dialog.addString("Please enter the number of the last file you wish to convert - count from the first file in the folder, ignoring any subfolders.","# here",5);

	Dialog.show();

	finish=Dialog.getString();

	inDir= getDirectory("Choose the folder to open.");

	outDir= getDirectory("Choose where to save the files.");



function processFolderFiles(inDir){

	files = getFileList(inDir);

	Array.print(files);

	for (i=start; i<finish; i++){

		if(File.isDirectory(files[i]))

		processFolder("" + inDir + files[i]);

		if(endsWith(files[i], filetype));

		filename = File.getName(files[i]);

		print(filename);

		processFile(inDir, outDir, files[i]);

		}
```

```
}

};


//For future feature - useful for immunofluorescence

Dialog.create("Image type?");

Dialog.addChoice("Are you converting single channel or multi-channel images? (multi-channel currently unusable)",newArray("Single", "Multi"));

Dialog.show();

channel=Dialog.getChoice;


print("Images will be imported as "+channel+"-channel images.");


//Allow specification of which channels to use

if(channel=="Multi"){

	colour= newArray("Blue", "Cyan", "Green", "Grey", "Magenta", "Red", "Yellow");


Dialog.create("Visible Channels");

	Dialog.addCheckboxGroup(3,3,colour, newArray(true, false, true, false, false, true, false));
```

```
	Dialog.show();
blue= Dialog.getCheckbox();
cyan= Dialog.getCheckbox();
green= Dialog.getCheckbox();
grey= Dialog.getCheckbox();
magenta= Dialog.getCheckbox();
red= Dialog.getCheckbox();
yellow= Dialog.getCheckbox();

//Set iteration to same length as number of channels - skip n files
iterator=blue+red+green+cyan+grey+magenta+yellow

function processFlourescence(inDir){
	files = getFileList(inDir);
	Array.print(files);
	for (i=0; i<files.length; i+=iterator){
		if(File.isDirectory(files[i]))
		processFolder("" + inDir + files[i]);
		if(endsWith(files[i], filetype))
```

```
                processFile(inDir, outDir, files[i])};


        setBatchMode(true);
        //continue fluorescence here
        }
}

if(channel=="Single"){

        if(Input=="Folder"){

//Don't open windows, just do in background
setBatchMode(true);
processFolder(inDir);
        }
}

if(channel=="Single"){
```

```
	if(Input=="Files"){


//Don't open windows, just do in background

setBatchMode(true);

processFolderFiles(inDir);

	}

}


//Define new function to iterate through files in folder and only for certain file types

//Change i initial value to change start point for conversion

function processFolder(inDir){

	files = getFileList(inDir);

	Array.print(files);

	for (i=0; i<files.length; i++){

		if(File.isDirectory(files[i]))

		processFolder("" + inDir + files[i]);

		if(endsWith(files[i], filetype));

		filename = File.getName(files[i]);

		print(filename);
```

```
                processFile(inDir, outDir, files[i]);
                }
}

//Define new function to open and name files
function processFile(inDir, outDir, file){
        if(seriesnum>1){
                if(series1==1){run("Bio-Formats Importer", "open=["+inDir+filename+"]
autoscale color_mode=Default rois_import=[ROI manager] view=Hyperstack
stack_order=XYCZT Series_1]");
                print("Processing:" +inDir+filename+" Series 1");
                saveAs(convert,outDir+filename+"Series_1");
                print("Saving "+file+"Series_1 to "+outDir);
                close();
                run("Collect Garbage");
                wait(500);
        };
```

```
if(series2==1){run("Bio-Formats Importer", "open=["+inDir+filename+"] autoscale
color_mode=Default rois_import=[ROI manager] view=Hyperstack stack_order=XYCZT
Series_2]");
        print("Processing:" +inDir+filename+" Series 2");
        saveAs(convert,outDir+filename+"Series_2");
        print("Saving "+file+"Series_2 to "+outDir);
        close();
        run("Collect Garbage");
        wait(500);
};
if(series3==1){run("Bio-Formats Importer", "open=["+inDir+filename+"] autoscale
color_mode=Default rois_import=[ROI manager] view=Hyperstack stack_order=XYCZT
Series_3]");
        print("Processing:" +inDir+filename+" Series 3");
        saveAs(convert,outDir+filename+"Series_3");
        print("Saving "+file+"Series_3 to "+outDir);
        close();
        run("Collect Garbage");
        wait(500);
```

```
};

if(series4==1){run("Bio-Formats Importer", "open=["+inDir+filename+"] autoscale
color_mode=Default rois_import=[ROI manager] view=Hyperstack stack_order=XYCZT
Series_4]");

        print("Processing:" +inDir+filename+" Series 4");

        saveAs(convert,outDir+filename+"Series_4");

        print("Saving "+file+"Series_4 to "+outDir);

        close();

        run("Collect Garbage");

        wait(500);

}

if(series5==1){run("Bio-Formats Importer", "open=["+inDir+filename+"] autoscale
color_mode=Default rois_import=[ROI manager] view=Hyperstack stack_order=XYCZT
Series_5]");

        print("Processing:" +inDir+filename+" Series 5");

        saveAs(convert,outDir+filename+"Series_5");

        print("Saving "+file+"Series_5 to "+outDir);

        close();

        run("Collect Garbage");
```

```
        wait(500);
    };
    if(series6==1){run("Bio-Formats Importer", "open=["+inDir+filename+"] autoscale
color_mode=Default rois_import=[ROI manager] view=Hyperstack stack_order=XYCZT
Series_6]");
        print("Processing:" +inDir+filename+" Series 6");
        saveAs(convert,outDir+filename+"Series_6");
        print("Saving "+file+"Series_6 to "+outDir);
        close();
        run("Collect Garbage");
        wait(500);
    };
    if(series7==1){run("Bio-Formats Importer", "open=["+inDir+filename+"] autoscale
color_mode=Default rois_import=[ROI manager] view=Hyperstack stack_order=XYCZT
Series_7]");
        print("Processing:" +inDir+filename+" Series 7");
        saveAs(convert,outDir+filename+"Series_7");
        print("Saving "+file+"Series_7 to "+outDir);
        close();
```

```
            run("Collect Garbage");
            wait(500);
        };
};

        else if (seriesnum==1){run("Bio-Formats Importer", "open=["+inDir+filename+"]
autoscale color_mode=Default rois_import=[ROI manager] view=Hyperstack
stack_order=XYCZT ["+seriesstring+"]");
print("Processing:" +inDir+filename);
        saveAs(convert,outDir+filename+seriesstring);
        print("Saving "+file+seriesstring+" to "+outDir);
        close();
        run("Collect Garbage");
        wait(500);
        };

};

//if(filetype==".vsi"){
```

```
        //run Bioformats

        //else

        //run("Convert...", "output_format=convert interpolation=None scale=1 read");

//}


//setBatchMode(true);

//processFiles(inDir, outDir, "");

//print("Done!");


//Excitement! YAY!

Dialog.create("Successfully finished macro run")

Dialog.addMessage("YAY!")

Dialog.show();

//run("Bio-Formats Importer",

//"open=[S:\\Research\\Respiratory Research Group\\Jo-

Maree\\Tricia\\BIProjectTake2\\BI01108.vsi] autoscale color_mode=Default

rois_import=[ROI manager] view=Hyperstack stack_order=XYCZT series_2");

//close();
```

Macro for automatically sampling selections

This macro was designed to iterate through a specified folder and open every file within it, before isolating pre-specified selections and saving them as individual RGB images. The selections had to be mapped and saved as a selection by ImageJ/FIJI prior to use, and the filepath for the selection criteria had to be accurately mapped. New files would be sequentially numbered beginning with '0' and increasing by 1 for each selection from the same image. Coding language is IJ1/FIJI native language.

```
//explanation dialog of macro

Dialog.create("Hi there.");

Dialog.addMessage("This is a macro for taking small images from a larger whole and saving them as RGB files. It can only handle three-channel images.");

Dialog.show();


//reminder to save ROIs somewhere

Dialog.create("Reminder.");

Dialog.addMessage("Remember to save the ROIs you want to use as selections somewhere and get the file path onto the clipboard.");

Dialog.show();


//enter file suffix to search for


Dialog.create("Starting file types")

Dialog.addString("What type of files are the original images?",".tif");

Dialog.show();


//stores file suffix

filetype = Dialog.getString();


//allows change of filetype if needed

Dialog.create("New images");

Dialog.addString("What filetype would you like to save the new images as? Please do not include the dot.","tiff");

Dialog.show();


//stores new file suffix

newtype = Dialog.getString();
```

```
//choose folder to search for images to convert

Dialog.create("Open which file?");

Dialog.addMessage("Please choose the folder which contains the images you would like to apply this macro to.");

Dialog.show();


inDir= getDirectory("Choose the folder to open.");


//choose save destination

Dialog.create("Save new files where?");

Dialog.addMessage("Please choose the folder where you would like to save the new files.")

Dialog.show();


outDir= getDirectory("Choose where to save the files.");


//Bit clunky but choose file containing ROIs required

Dialog.create("ROI files");

Dialog.addString("Please enter the full path of the file which contains the ROIs you wish to use.","FILEPATHHERE");

Dialog.show();


ROIpath = Dialog.getString();


//Enter max iteration value to limit iterations (backup programming only)

Dialog.create("Number of selections");

Dialog.addNumber("How many selections are you saving per image?",16);

Dialog.show();


ROITotal = Dialog.getNumber;
```

```
//In case of crashing due to memory, allows easy start at any file
Dialog.create("Start point");
Dialog.addNumber("If this program was interrupted or ran out of memory, please enter the value shown after the last file was closed in order to restart in the same position.",0);
Dialog.show();

ires = Dialog.getNumber();

//Stop it opening new windows; run macro in background
setBatchMode(true);
processFolder(inDir);

//set function to go through folder
function processFolder(inDir){
	files = getFileList(inDir);
	Array.print(files);
	for (i=ires; i<files.length; i++){
		if(File.isDirectory(files[i]))
		processFolder("" + inDir + files[i]);
		if(endsWith(files[i], filetype));
		filename = File.getName(files[i]);
		processFile(inDir, outDir, files[i]);
		}
}	//processFolder iteration

//poss obsolete, reset ROI start point
ROINum = 0
```

```
//set function to process files
function processFile(inDir, outDir, file){
	roiManager("Open", ROIpath);
	print("Opening: "+filename);
	open(inDir+filename);
		for(ROINum=0;ROINum<ROITotal;ROINum++) {
			roiManager("Select", ROINum);
			print("Duplicating selection "+ROINum+" now.");
			run("Duplicate...", "title=["+filename+"-"+ROINum+"] duplicate
channels=1-3");
			title = filename+"-"+ROINum;
			print("Converting to RGB.");
			run("RGB Color");
			print("Saving "+title+" now.");
			saveAs(newtype,outDir+title);
			print("Closing RGB image now.");
			close();
			print("Closing duplicate "+ROINum);
			close();
			print("Collecting garbage...");
			run("Collect Garbage");
			wait(500);
		};	// end ROI number iteration
		print("Closing "+filename);
		close();
		print(i+1);
		print("Collecting garbage...");
		run("Collect Garbage");
		wait(1000);
```

```
        };      // end processFile iteration


//Excitement! YAY!
Dialog.create("All done!");
Dialog.addMessage("Yay! All of your files have been processed!");
Dialog.show();
```

Macro for allowing rapid semi-automated analysis of immunocytochemistry images

This macro was initially written/coded by Dr. Jo-Maree Courtney for use in this study, however some alterations were made by the study author, which are noted in the code annotations.

This macro was designed to open five images at random from each field of view/region of interest within a specified folder. The macro allows the user to count the number of nuclei visible in the image manually, and then allows the user to utilise the 'threshold' function to denote areas of staining. The number of nuclei, image name and number, the number of the region of interest (in this case the well number on the slide, SEE METHODS) and both the total area stained and the area stained/nuclei were then output in simple layout for ease of analysis. Coding language is FIJI native language.

```
// JUST NEED TO ADD THE ITERATION TO THE WELL NUMBER!!!!


requires("1.33s");
dir = getDirectory("Choose a Directory ");
setBatchMode(false);
dirStructure = split(dir, "\\/");
folder = dirStructure[lengthOf(dirStructure)-1];
list = getFileList(dir);

//SET UP TABLE
tableTitle=folder;
tableTitle2="["+tableTitle+"]";
run("Table...", "name="+tableTitle2+" width=1000 height=250");
//tableTitle="["+folder+"}";
//run("Table...", "name="+tableTitle+" width=1000 height=250");
print(tableTitle2, "\\Headings:Slide\tWell\tImage\tCell Count\tArea\tArea per cell");

for (n=0; n<9; n++) {

	//GENERATE RANDOM NUMBER ARRAYS
	selection = newArray(16);
	while (occurencesInArray(selection, 1)<5) {
		pick = round(random*15);
		selection[pick] = 1;
	}

	for (i=0; i<list.length; i++) {
		file = list[i];
```

```
        filepath = dir + list[i];
        slide = "unknown";
        well = "unknown";

    if (endsWith(file, ".tif")) {  // DO EVERYTHING WITHIN THIS SECTION

      if (startsWith(file, "BIT")) {  // GETTING SLIDE AND WELL DETAILS
FROM BI AND BIT FILES
        //get slide number
        slide = substring(file, 3, 6);
                    //get well number
                    subs = substring(file, 3, 7);
        if (endsWith(subs, "_"))
            well = substring(file, 7, 9);
                    else
            well = substring(file, 6, 8);
      }
      else if (startsWith(file, "BI")) {
        //get slide number
        slide = substring(file, 2, 5);
                    //get well number
                    subs = substring(file, 2, 6);
        if (endsWith(subs, "_"))
            well = substring(file, 6, 8);
                    else
            well = substring(file, 5, 7);
      }
      else {
        print("Not a BI file");
```

```
            exit("Not a BI or BIT file");
        } // END OF GETTING SLIDE AND WELL INFO

        //get image number
            endName = substring(file, lengthOf(file)-6);
            if (startsWith(endName, "-"))
                image = substring(endName, 1, 2);
            else
                image = substring(endName, 0, 2);

                wellNum = parseInt(well);
                imgNum = parseInt(image);

                if (wellNum == n && selection[imgNum] == 1) {
                    print(file);

                    // DO ANALYSIS
============================================================
                    open(filepath);
                    name = File.nameWithoutExtension;
                    //dir = getInfo("image.directory");
                    rename("image");

                    // DECONVULUTE IMAGE FIRST TO GET UNIMPEDED
BROWN STAIN THEN GO BACK TO ORIGINAL FOR NUCLEI COUNT
                    selectWindow("image");
                    // SET SCALE EDIT - TRICIA
                    run("Set Scale...", "distance=2.9034 known=1 pixel=1.000
unit=micron global");
                    run("Colour Deconvolution", "vectors=[H DAB] hide");
```

```
selectWindow("image-(Colour_3)");
run("Close");
selectWindow("image-(Colour_1)");
run("Close");

// COUNT NUCLEI MANUALLY

selectWindow("image");
setTool("multipoint");
waitForUser("Select all the nuclei");
run("Clear Results");
run("Measure");

nuclei = nResults;

selectWindow("Results");
run("Close");

//GET TOTAL AREA OF BROWN STAIN
selectWindow("image-(Colour_2)");
setAutoThreshold("Minimum");
run("Threshold...");
waitForUser("Is this threshold ok? Adjust if need be.");

run("Create Selection"); //EDIT BY TRICIA
run("Set Measurements...", "area area_fraction display
redirect=None decimal=3");
run("Measure");
```

```
                                area = getResult("Area");
                                selectWindow("Results");
                                run("Close");

                                areaPerCell = area/nuclei;

                                print(tableTitle2,
slide+"\t"+well+"\t"+image+"\t"+nuclei+"\t"+area+"\t"+areaPerCell);

                                run("Close All");

                                // END OF ANALYSIS
=========================================================

                        }

            } // END OF IF TIFF STATEMENT

        }  // END OF FILE ITERATION LOOP

} // END OF WELL ITERATION LOOP

//Returns the number of times the value occurs within the array
function occurencesInArray(array, value) {
   count=0;
   for (a=0; a<lengthOf(array); a++) {
      if (array[a]==value) {
         count++;
      }
```

```
}
return count;
```

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*

Appendix 2: Dissociation curves and isotype controls

This appendix demonstrates the appearance of a clean dissociation curve for qPCR and a non-staining isotype control for immunocytochemical analysis. The dissociation curves (Figure 8.1-1) demonstrated smooth dissociation and a single pronounced peak, indicating specific primer binding and target amplification. The isotype control staining, which used mouse IGg1 in place of primary antibody, showed that the secondary antibodies did not bind non-specifically to the cells

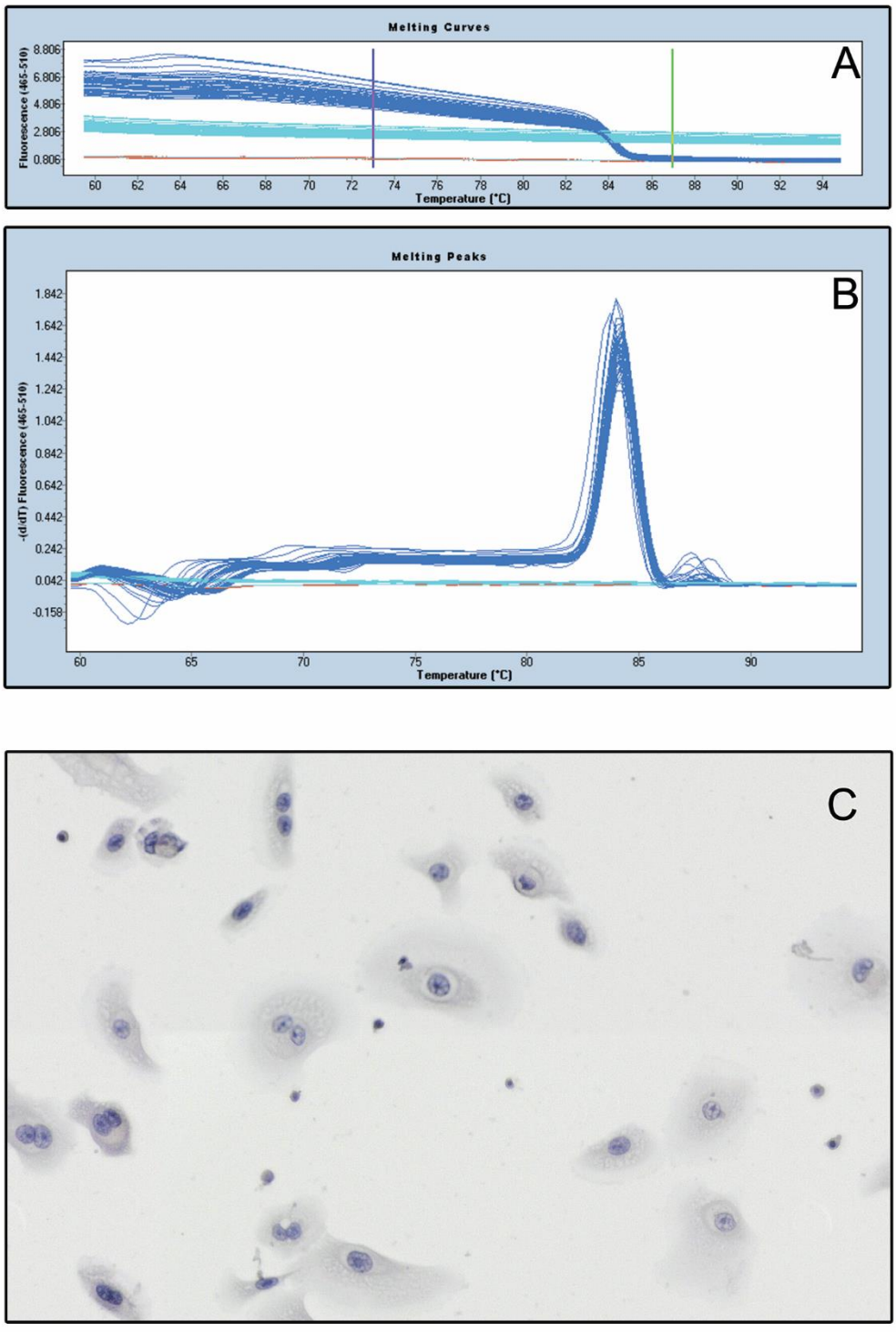

Figure 8.1-1. Dissociation (melting) curves for qPCR and isotype control for immunocytochemistry. ***A and B*** *show qPCR melting curves for a primer set with smooth dissociation and a single pronounced peak, indicating specific primer binding and target amplification.* ***C*** *demonstrates isotype control 'staining', showing that the secondary antibodies did not bind non-specifically to the cells.*

Useful abbreviations

NNS *– non-smokers*
NLFS *– smokers with normal lung function*
COPD-CS *– current smokers with airflow limitation*
COPD-ES *– ex-smokers with airflow limitation*
LABA/LAMA *– long-acting β-agonist/muscarinic antagonist*
pHBECs *– primary human bronchial epithelial cells*
TGF-β *– transforming growth factor-β1*
CSE *– cigarette smoke extract*